Check for updates

EFSA Journal

SCIENTIFIC OPINION

ADOPTED: 29 September 2021

doi: 10.2903/j.efsa.2021.6611

In vivo and *in vitro* random mutagenesis techniques in plants

EFSA Panel on Genetically Modified Organisms (GMO),
Ewen Mullins, Jean-Louis Bresson, Tamas Dalmay, Ian Crawford Dewhurst,
Michelle M Epstein, Leslie George Firbank, Philippe Guerche, Jan Hejatko,
Francisco Javier Moreno, Hanspeter Naegeli, Fabien Nogué, Jose Juan Sánchez Serrano,
Giovanni Savoini, Eve Veromann, Fabio Veronesi, Josep Casacuberta, Paolo Lenzi,
Irene Munoz Guajardo, Tommaso Raffaello and Nils Rostoks

Abstract

Mutations are changes in the genetic material that may be transmitted to subsequent generations. Mutations appear spontaneously in nature and are one of the underlying driving forces of evolution. In plants, *in vivo* and *in vitro* random mutagenesis relies on the application of physical and chemical mutagens to increase the frequency of mutations thus accelerating the selection of varieties with important agronomic traits. The European Commission has requested EFSA to provide a more detailed description of *in vivo* and *in vitro* random mutagenesis techniques and the types of mutations and mechanisms involved, to be able to conclude on whether *in vivo* and *in vitro* random mutagenesis techniques are to be considered different techniques. To address the European Commission request, a literature search was conducted to collect information on the random mutagenesis techniques used in plants both *in vivo* and *in vitro*, on the type of mutations generated by such techniques and on the molecular mechanisms underlying formation of those mutations. The GMO Panel concludes that most physical and chemical mutagenesis techniques have been applied both *in vivo* and *in vitro*; the mutation process and the repair mechanisms act at cellular level and thus there is no difference between application of the mutagen *in vivo* or *in vitro*; and the type of mutations induced by a specific mutagen are expected to be the same, regardless of whether such mutagen is applied *in vivo* or *in vitro*. Indeed, the same mutation and the derived trait in a given plant species can be potentially obtained using both *in vivo* and *in vitro* random mutagenesis and the resulting mutants would be indistinguishable. Therefore, the GMO Panel concludes that the distinction between plants obtained by *in vitro* or *in vivo* approaches is not justified.

© 2021 European Food Safety Authority. *EFSA Journal* published by John Wiley and Sons Ltd on behalf of European Food Safety Authority.

Keywords: random mutagenesis, *in vivo*, *in vitro*, chemical mutagenesis, physical mutagenesis, mutagen, mutation

Requestor: European Commission

Question number: EFSA-Q-2020-00445

Correspondence: GMO_applications@efsa.europa.eu

Panel members: Ewen Mullins, Jean-Louis Bresson, Tamas Dalmay, Ian Crawford Dewhurst, Michelle M Epstein, Leslie George Firbank, Philippe Guerche, Jan Hejatko, Francisco Javier Moreno, Hanspeter Naegeli, Fabien Nogué, Nils Rostoks, Jose Juan Sánchez Serrano, Giovanni Savoini, Eve Veromann and Fabio Veronesi.

Declarations of interest: The declarations of interest of all scientific experts active in EFSA's work are available at https://ess.efsa.europa.eu/doi/doiweb/doisearch.

Acknowledgements: The GMO Panel wishes to thank the following for the support provided to this scientific output: the Molecular Characterisation Working Group of the GMO Panel and the EFSA staff Laura Martino.

Suggested citation: EFSA GMO Panel (EFSA Panel on Genetically Modified Organisms), Mullins E, Bresson JL, Dalmay T, Dewhurst IC, Epstein MM, Firbank LG, Guerche P, Hejatko J, Moreno FJ, Naegeli H, Nogué F, Sánchez Serrano JJ, Savoini G, Veromann E, Veronesi F, Casacuberta J, Lenzi P, Munoz Guajardo I, Raffaello T and Rostoks N, 2021. *In vivo* and *in vitro* random mutagenesis techniques in plants. EFSA Journal 2021;19(11):6611, 30 pp. https://doi.org/10.2903/j.efsa.2021.6611

ISSN: 1831-4732

© 2021 European Food Safety Authority. *EFSA Journal* published by John Wiley and Sons Ltd on behalf of European Food Safety Authority.

This is an open access article under the terms of the Creative Commons Attribution-NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited and no modifications or adaptations are made.

The EFSA Journal is a publication of the European Food Safety Authority, a European agency funded by the European Union.

Summary

Mutations are changes in the genetic material that may be transmitted to subsequent generations. Mutations appear spontaneously in nature and are one of the underlying forces driving evolution and allowing the adaptation to an ever-changing environment. In plants, *in vivo* and *in vitro* random mutagenesis relies on the application of physical and chemical mutagens to plant material to artificially increase the number of mutations and expand the genetic variability which can be exploited in breeding programs.

This Opinion addresses four requests from the European Commission as described in the terms of reference:

1) To provide a more detailed description of random mutagenesis techniques as applied *in vivo* and *in vitro*.
2) To assess whether the types of genetic modification induced by random mutagenesis techniques are different depending on whether the technique is applied *in vivo* or *in vitro*.
3) To assess whether the molecular mechanism underlying formation of new mutations induced by random mutagenesis techniques is different if the techniques are applied *in vivo* or *in vitro*.
4) To assess whether *in vitro* random mutagenesis techniques are to be considered as different techniques compared to *in vivo* random mutagenesis or on the contrary, if they are to be considered as a continuum.

A protocol was developed following a low extent of planning according to 'Draft framework for protocol development for EFSA's scientific assessments' (EFSA, 2020). Each ToR was translated into scientifically answerable assessment questions and a literature search was conducted. Considering the time constraints to develop the document and that the field of plant random mutagenesis is not evolving at a fast pace, the literature search was limited to review papers and books/book chapters. The literature search results were evaluated independently by two EFSA scientific officers to retain documents which included information related to the type of random mutagenesis technique used in plants both *in vivo* and *in vitro*, the specific type of mutation generated by the random mutagenesis techniques and the molecular mechanisms leading to a certain mutation.

Based on the review of the information retrieved by the literature search and the experts' knowledge, the GMO Panel concluded that:

- different physical and chemical mutagenesis techniques have been applied both *in vivo* and *in vitro* and all of them can, in principle, be applied to both settings;
- the process and the repair mechanisms that are triggered by the mutagen act at the cellular level and thus there is no difference in the way the mutagen will affect the DNA whether the mutagen is applied *in vivo* or *in vitro*;
- the type of mutations induced by a specific mutagen are expected to be the same regardless of whether such mutagen is applied *in vivo* or *in vitro*.

The distinction between plants obtained by *in vitro* or *in vivo* approaches is, therefore, not justified. Indeed, the same mutation and the derived trait in a given plant species can be potentially obtained using both *in vivo* and *in vitro* random mutagenesis and the resulting mutants would be indistinguishable.

Table of contents

Abstract 1
Summary 3
1. Introduction 5
1.1. Background as provided to EFSA by European Commission 5
1.2. Background as provided by EFSA 5
1.3. Terms of Reference 5
2. Data and methodologies 6
3. Protocol 6
3.1. Problem formulation 6
3.2. Extent of planning 6
3.3. Definition of methods 7
3.3.1. Literature search 7
3.3.2. Screening of the literature search results 7
3.3.3. Consultation 7
4. Assessment 8
4.1. Introduction 8
4.1.1. Spontaneous and induced mutations in the context of plant breeding 8
4.1.2. Historical view on random mutagenesis in mutation breeding 9
4.2. Addressing ToR1: To provide a more detailed description of random mutagenesis techniques as applied *in vivo* and *in vitro* 10
4.2.1. What random mutagenesis techniques are used to obtain mutant plants? 10
4.2.1.1. Mutation breeding: summary of the main steps 10
4.2.1.2. General considerations on *in vivo* and *in vitro* random mutagenesis techniques in plants 11
4.2.1.3. Physical mutagenesis techniques applied in mutation breeding 12
4.2.1.4. Chemical mutagenesis techniques applied in mutation breeding 13
4.2.1.5. Examples of *in vivo* and *in vitro* random mutagenesis applications in plants 14
4.2.2. Are all these techniques applicable both *in vivo* and *in vitro*? 16
4.3. Addressing ToR3: To assess whether the molecular mechanism underlying random mutagenesis techniques is different if the techniques are applied *in vivo* or *in vitro* 16
4.3.1. What are the underlying molecular mechanisms which generate the mutations? 16
4.3.1.1. Mechanisms leading to DNA damage 16
4.3.1.2. Mechanisms leading to repair 17
4.3.2. Is there any difference between these molecular mechanisms whether they happen *in vivo* or *in vitro*? 19
4.4. Addressing ToR2: To assess whether the types of genetic modification induced by random mutagenesis techniques are different depending on whether the technique is applied *in vivo* or *in vitro* 19
4.4.1. What type of alterations at the DNA level are induced by random mutagenesis? 19
4.4.1.1. Types of mutations 20
4.4.2. Is there any difference between the mutations whether they are obtained *in vivo* or *in vitro*? 20
4.5. Addressing ToR4: To assess whether *in vitro* random mutagenesis techniques are to be considered as different techniques compared to *in vivo* random mutagenesis techniques or on the contrary, if they are to be considered as a *continuum* 20
4.5.1. Are *in vitro* and *in vivo* random mutagenesis techniques considered to be different or not? 20
5. Conclusions 20
References 21
Abbreviations 24
Glossary 25
Appendix A – Literature searches. Search strategies 26

1. Introduction

1.1. Background as provided to EFSA by European Commission

The judgment of the Court of Justice of the European Union (CJEU) in Case C-528/16[1] on mutagenesis held that Article 3(1) of Directive 2001/18 on the deliberate release of Genetically Modified Organisms (OGM)[2] must be interpreted as meaning that "only GMOs obtained by means of techniques/methods of mutagenesis which have conventionally been used in a number of applications and have a long safety record" are excluded from the scope of that directive. The CJEU in its reasoning referred to the "application of conventional methods of random mutagenesis" without distinguishing further between *in vivo* and *in vitro* random mutagenesis and distinguished them from "new techniques/methods of mutagenesis which have appeared or have been mostly developed since Directive 2001/18 was adopted".[3]

Following the ruling of the CJEU, the Conseil d'Etat of France issued on 7 February 2020 a judgment on organisms obtained by mutagenesis. In its judgment, the Conseil d'Etat describes conventional or random mutagenesis as a technique triggering random mutations in a DNA sequence through the action of chemical or physical mutagens. The French Conseil d'Etat distinguishes between *in vivo* and *in vitro* random mutagenesis techniques. *In vivo* random mutagenesis would consist in the application of chemical or physical mutagens to whole plants or parts of plants, which would then be subject to selection procedures in order to identify the interesting mutations. *In vitro* random mutagenesis would consist in subjecting plant cells to chemical or physical mutagenic agents. The modified cells would then be subject to techniques of *in vitro* cell culture in order to regenerate the whole plant.

EFSA, in its Scientific opinion addressing the safety assessment of plants developed using Zinc Finger Nuclease 3 and other Site-Directed Nucleases with similar function,[4] examines conventional plant breeding techniques relevant for a comparison with Site Directed Nuclease-3 technique. Among these conventional techniques, EFSA describes mutation breeding by chemical and physical mutagenesis. While EFSA explains the various modes of action depending on the chemical mutagens or the type of radiation used, the Authority makes no distinction between the application of the techniques *in vitro* or *in vivo*.

Member States have never made a distinction between *in vitro* and *in vivo* either when implementing the seed legislation, the plant propagating material legislation or the GMO legislation.

It is therefore important to provide a robust scientific understanding of random mutagenesis techniques and a robust scientific analysis as to whether the distinction between *in vitro* and *in vivo* is scientifically justified.

1.2. Background as provided by EFSA

Following a request from the European Commission (Ref. Ares(2020)2651289 - 20/5/2020), EFSA assigned the mandate to the molecular characterisation working group (MC WG) of the GMO Panel in May 2020 (Ref. BU/GdS/EW/svh - OC-2020-23489581).

1.3. Terms of Reference

Against this background, the Commission asks EFSA, in accordance with Art 29 of Regulation (EC) No 178/2002:

A) To provide a more detailed description of random mutagenesis techniques as applied *in vivo* and *in vitro*.
B) To assess whether the types of genetic modification induced by random mutagenesis techniques are different depending on whether the technique is applied *in vivo* or *in vitro*.
C) To assess whether the molecular mechanism underlying random mutagenesis techniques is different if the techniques are applied *in vivo* or *in vitro*.

[1] Case C-528/16, *Confédération paysanne and Others*, Judgment of 25 July 2018, EU:C:2018:583.
[2] Directive 2001/18/EC of the European Parliament and of the Council on the deliberate release into the environment of genetically modified organisms and repealing Council Directive 90/220/EEC. OJ L 106, 17.4.2001, p. 1. Article 4.
[3] Case C-528/16, *Confédération paysanne and Others*, Judgment of 25 July 2018, EU:C:2018:583, points 48 et 51.
[4] EFSA GMO Panel (EFSA Panel on Genetically modified organisms), 2012. Scientific opinion addressing the safety assessment of plants developed using Zinc Finger Nuclease 3 and other Site-Directed Nucleases with similar function. EFSA Journal 2012;10 (10):2943, 31 pp. https://doi.org/10.2903/j.efsa.2012.2943. Available online: www.efsa.europa.eu/efsajournal

D) To assess whether *in vitro* random mutagenesis techniques are to be considered as different techniques compared to *in vivo* random mutagenesis techniques or on the contrary, if they are to be considered as a *continuum*.

2. Data and methodologies

To address the four terms of reference, the MC WG followed a low extent of planning developed according to the 'Draft framework for protocol development for EFSA's scientific assessments' (EFSA, 2020) (Section 3). It should be noted that according to the background information and the ToRs provided by the European Commission, the MC WG was not mandated to provide risk assessment considerations on *in vivo* and *in vivo* random mutagenesis techniques in plants.

3. Protocol

3.1. Problem formulation

The ToRs were translated into scientifically answerable assessment questions in line with the EFSA draft document on protocol development for non-application mandates (Step 1.1 and Step 1.2; EFSA, 2020). Each ToR was translated into question(s) as detailed below:

A) ToR1: To provide a more detailed description of random mutagenesis techniques as applied *in vivo* and *in vitro*.

   a) What random mutagenesis techniques are used to obtain mutant plants?
   b) Are all these techniques applicable both *in vivo* and *in vitro*?

B) ToR2: To assess whether the types of genetic modification induced by random mutagenesis techniques are different depending on whether the technique is applied *in vivo* or *in vitro*.

   a) What type of alterations at the DNA level are induced by random mutagenesis?
   b) Is there any difference between the type of alterations induced *in vivo* or *in vitro*?

C) ToR3: To assess whether the molecular mechanism underlying random mutagenesis techniques is different if the techniques are applied *in vivo* or *in vitro*.

   a) What are the underlying molecular mechanisms which generate the mutations?
   b) Is there any difference between these molecular mechanisms whether they happen *in vivo* or *in vitro*?

D) ToR4: To assess whether *in vitro* random mutagenesis techniques are to be considered as different techniques compared to *in vivo* random mutagenesis techniques or on the contrary, if they are to be considered as a *continuum*.

   a) Are *in vitro* and *in vivo* random mutagenesis techniques considered to be different or not?

It should be noted that the GMO Panel was not mandated to develop new definitions of '*in vivo* and *in vitro* random mutagenesis'. In the context of this scientific opinion, the definition of '*in vivo* and *in vitro* random mutagenesis' was provided by the European Commission in the background information of the mandate (Section 1.1) and it is reported below:

'*In vivo* random mutagenesis would consist in the application of chemical or physical mutagens to whole plants or parts of plants, which would then be subject to selection procedures in order to identify the interesting mutations. *In vitro* random mutagenesis would consist in subjecting plant cells to chemical or physical mutagenic agents. The modified cells would then be subject to techniques of *in vitro* cell culture in order to regenerate the whole plant'.

3.2. Extent of planning

In order to answer the formulated research questions (Section 3.1), the MC WG decided to follow a qualitative approach consisting in a literature search (Step 1.3; EFSA, 2020). Given the time constraints to develop the scientific opinion and considering that random mutagenesis techniques in plants are not evolving at a fast pace, the literature search was limited to review and book chapters (Step 2; EFSA, 2020).

3.3. Definition of methods

3.3.1. Literature search

The MC WG with the support of an information specialist designed literature searches to identify possible books, chapter of books or reviews published on random mutagenesis techniques as applied *in vivo* and *in vitro* in plants. The GMO panel considered that taking into account very extensive history of random mutagenesis and available original research, it is reasonable to restrict the search to book chapters and reviews. The search was not limited by publication year.

The bibliographic databases listed in Table 1 were searched in order to identify relevant studies. The databases have been identified in line with the defined scope of the review.

Table 1: Bibliographic databases searched for relevant studies

Source of information	Platform
PubMed	Web of Science
Scopus	Scopus.com
Web of Science Core Collection • Science Citation Index Expanded (SCI-EXPANDED) – 1975–present • Conference Proceedings Citation Index- Science (CPCI-S) – 1990–present • Book Citation Index – Science (BKCI-S) – 2005–present • Emerging Sources Citation Index (ESCI) – 2005–present • Current Chemical Reactions (CCR-EXPANDED) – 1985–present • (Includes Institut National de la Propriete Industrielle structure data back to 1840) • Index Chemicus (IC) – 1993–present	Web of Science

The search strings as run in the sources of information are available in the Appendix. They are structured as a combination of three searches: search 1 combines terms for chemical or physical mutagenesis and plants; search 2 combines terms for random mutagenesis and plants; search 3 combines terms for mutagenesis in plants and generic terms for techniques. Keywords have been selected with the help of the members of the WG, consulting thesaurus (e.g. CAB Thesaurus) and dictionaries.

The searches are limited to documents published in the English language and to books, book chapters or reviews. When the bibliographic databases did not have filter for reviews, i.e. CAB Abstracts, a search string was designed to identify this type of publication.

Searches were run on 22 December 2020: 344 documents were retrieved in CAB Abstracts, 215 in Scopus and 202 in the databases of the Web of Science Core Collection. The output of the searches, that is, records retrieved from bibliographic databases was exported into an EndNote library (Clarivate Analytics). After removing the duplicate references in EndNote, 517 unique documents were identified by the databases.

3.3.2. Screening of the literature search results

The 517 unique documents were screened independently by two EFSA scientists by title and abstract to retain only the relevant papers and books which provide information on at least one of the following aspects:

- the type of random mutagenesis technique used in plants both *in vivo* and *in vitro*;
- the specific type of mutation generated by the random mutagenesis techniques;
- the molecular mechanism(s) leading to a certain mutation.

The result of the two independent screenings were compared, and ambiguities were resolved. The final list consists of 294 relevant documents. During the writing process, the experts deemed it necessary to add some additional references not included in the initial literature search results, in order to provide further support to the topics described in the scientific opinion.

3.3.3. Consultation

In line with its policy on openness and transparency, EFSA consulted EU Member States and its stakeholders via an online public consultation. Between May and June 2021, interested persons were

invited to submit their comments on the draft GMO Panel Scientific Opinion.[5] Following this consultation process, the document was revised by the GMO Panel and the experts of the MC WG.

The outcome of the online public consultation is reported in an EFSA Technical report that will be published on EFSA's website together with the final Scientific Opinion.

4. Assessment

4.1. Introduction

All living organisms encode in their genome the instructions for growth and development. Genomes accumulate mutations over time, which are changes in the genetic material that may be passed to the offspring. Hugo de Vries was the first scientist introducing the concept of 'mutation' at the beginning of the 20th century (Till et al., 2018). Spontaneous mutations, which are essentially random, are one of the powerful forces driving the evolution which result in new individuals and may lead to new genera and species forming all the kingdoms of life (Mba et al., 2010; Riaz and Gul, 2015). In the plant kingdom, this genetic variation has allowed plants to adapt to a wide range of environmental conditions, among many other benefits (Bado et al., 2015).

The DNA molecule consists of two strands that wind around one another to form a shape known as a double helix. Each strand has a backbone made of alternating sugar molecules (deoxyribose) and phosphate groups. Attached to each sugar is one of the four bases, adenine (A), cytosine (C), guanine (G) and thymine (T). A mutation in the DNA is usually triggered by an initial modification to a base, to the bond between the base and the sugar, or to the phosphodiester bond between nucleotides. Cells deploy a series of molecular mechanisms in the attempt to repair such modifications. When the recruited specialised enzymes, involved in the repair mechanisms, are not able to revert the change to its original conformation, the change becomes stable and it is called 'mutation'.

4.1.1. Spontaneous and induced mutations in the context of plant breeding

Spontaneous mutations are changes in the DNA that are not caused by human intervention, whereas induced mutations are caused by mutagenic agents brought by humans. Common sources of spontaneous mutations include for example errors in the DNA replication process or reactive oxygen species (ROS) that causes genetic alterations. The presence in the genome of mobile genetic elements such as transposons is another source of mutations, both due to their repetitive nature and their mobile capacity. The exposure of the plant to various physical conditions, such as UV light, or to naturally present chemical or biological agents could also trigger the occurrence of mutations. Mutations are the basis of the genetic variability we observe in all the living organisms (Bado et al., 2015). The frequency of spontaneous mutations depends on the environment. In particular, cultivating plants *in vitro* frequently increases the rate of appearance of spontaneous mutation, a phenomenon frequently known as 'somaclonal variation' (Larkin and Scowcroft, 1981). Both the particular environmental conditions of the *in vitro* culture settings, and the tissue reprograming, and disorganised growth frequently associated to *in vitro* culture, particularly when callus formation is involved, increase the rate of accumulation of spontaneous mutations (Bairu et al., 2011). Since the dawn of agriculture, farmers have used spontaneous mutations as source of genetic diversity. Plant breeding has exploited this natural genetic variability to select phenotypes with desired agronomic characteristics, such as the abolishment of pod or head shattering, spike dehiscence in grasses, the loss of bitterness in species like eggplants, cabbage or parthenocarpy in bananas and grapes (Mba, 2013). The increased rate of mutations associated with *in vitro* culture has also been used in breeding programs. Regenerating plants from *in vitro* culture has allowed the selection of some varieties with, for example, improved plant architecture, pathogen resistance and abiotic stress tolerance (Bairu et al., 2011). However, irrespective of frequency of mutation, the variability present in natural populations for a particular trait is limited and the desired phenotype may, in most cases, not be present.

Therefore, from the beginning of the 20th century, plant breeders started to apply physical and chemical mutagenesis to induce mutations, in order to artificially increase plant genetic diversity in breeding programs, a process which is called mutation breeding (Kharkwal, 2012). In other words, mutation breeding consists of increasing the genetic variability of plant species of agronomic interest by inducing mutations at a higher frequency compared to spontaneous processes. Mutation breeding has contributed to bring to the market thousands of plant varieties with enhanced phenotypic

[5] Published at https://connect.efsa.europa.eu/RM/s/publicconsultation2/a0l1v00000D7rSW/pc0011

characteristics such as agronomic performance, nutritional characteristics, resistance to diseases, tolerance to environmental stress, resistance to herbicides and many others. In addition, more recent developments in plant breeding have resulted in new techniques for targeted mutagenesis in plants. Some of these techniques have been discussed in EFSA recent opinions (EFSA GMO Panel, 2020).

4.1.2. Historical view on random mutagenesis in mutation breeding

The possibility to artificially induce mutations in organisms to a higher frequency compared to the level of spontaneous mutations was described at the beginning of 20th century. Already at the end of the 19th century, the discovery of X-rays in 1895 by Röentgen, of radioactivity by Becquerel in 1896, and of radioactive elements by Marie and Pierre Curie in 1898 has paved the way for the use of atomic energy in plant breeding. In this regard, the work of Hermann Muller, who described the use of X-rays to mutagenise *Drosophila melanogaster*, was pioneering since he demonstrated that the mutation rate could be increased by treating sperm in male flies with X-rays (Muller, 1927; Kharkwal, 2012). In the same years, Lewis John Stadler published his work which demonstrated that the application of X-rays could induce mutations also in important crops such as maize (*Zea mays*) and barley (*Hordeum vulgare*) (Stadler, 1928). Stadler demonstrated not only that irradiating soaked seeds could increase the amount of mutations compared to dry seeds but also that the mutation rate is linearly dependent on the dosage of the physical mutagen applied (Kharkwal, 2012). In the 1940s, more scientific evidences were accumulated regarding the mutagenic effects of electromagnetic radiation in plants. It is worth mentioning that the first commercialised crop variety produced by irradiation was tobacco Chlorina which displayed a better leaf quality for an improved cigar production (Konzak, 1957).

Although scientific evidences on the effect of chemical mutagens started to emerge already at the beginning of the 20th century, the use of chemical mutagenesis to increase the frequency of genetic mutations became more systematic only in the 1940s (Gustafsson, 1960). Systematic investigation of the effect of chemicals started with Baur and Stubbe. While Baur treated shoot-tips of *Antirrhinum* spp. with various chemicals to study their capacity to induce mutations, Stubbe explored the mutagenic effects of a much broader range of chemicals demonstrating that some of them could indeed increase the mutation rate. The work of Auerbach and Rapoport provided fundamental understanding of the mutagenic effects of several chemicals on *D. melanogaster*, especially alkylating chemical agents like 1,5-dichloro-3-thiapentane. Rapoport, in particular, provided data on the mutagenic effect of more than 50 chemicals which have been extensively used in plant mutagenesis (Sen, 1951; Kharkwal, 2012; Leitao, 2012). The application of chemical mutagenesis to staple crops was soon after demonstrated by Gustafsson and co-workers who investigated the mutagenic effects of alkylating agents in barley (Gustafsson, 1960).

In Europe up to the 1950s, most of the work in plant mutagenesis was performed in Sweden and Germany with the research carried out by Gustafsson, Freisleben and Lein in barley (Konzak, 1957). The application of random mutagenesis techniques in barley was particularly important between the 1950s and 1970s thanks to the Swedish mutation programme led by Gustafsson. The work allowed the selection of barley varieties expressing important agronomic traits such as chlorophyll characters, short stature, stiffness of straw and dense ears (Kharkwal, 2012; Lundqvist, 2014). In the middle of the 20th century, plant mutagenesis was mainly performed using physical mutagens, with the application of chemical mutagenesis still limited. Mutational breeding was not considered yet a good method for crop improvement since the artificial induction of mutations by physical and chemical agents was considered having strong destructive effects on the plant material (Mac Key, 1956). In those years, scientists were also reflecting on whether artificially induced mutations were different from spontaneous ones and whether the process would be economically enough to be exploited for plant breeding (Mac Key, 1956; Konzak, 1957). In the 1950s, the precise nature of mutations was not fully understood yet. These years have been rather devoted to improving the application of mutagenesis techniques, in particular regarding the choice between physical or chemical agents and the determination of optimal doses. The effects of the different mutagenic agents were investigated based only on their effect on the physical structure of chromosomes (Konzak, 1957). In the 1960s, it started to be evident that chemical mutagens and physical irradiation can produce different types of chromosomal alterations (Gustafsson, 1960). Also, data started to emerge on the fact that physical and chemical mutagens do not possess the same mutagenic power, some gene loci react in a different way, and a combination of both chemical and physical mutagens could improve the performance of mutation breeding in a given species (Ehrenberg, 1960).

In the period 1950–1970, not only US and European countries but also Argentina, former Soviet Union, India, China and Japan, started ambitious mutation breeding programmes in plants. For example, at the beginning of 1950s in Japan, rice was the first agronomically important crop subjected to mutational breeding using different sources of radiation (Matsuo, 1962). In China, mutation breeding in foxtail millet, an important regional cereal, started in 1963 using gamma radiations and become widely used to generate mutations in foxtail millet over the following 20 years (Diao and Jia, 2017). In US, evidence that the DNA damage caused by X-ray radiation could be exploited to transfer disease resistance from a wild variety into a cultivated one emerged when some experiments demonstrated that leaf rust resistance from *Aegilops umbellulata* could be introduced in wheat (*Triticum aestivum*) (Konzak, 1957). In Argentina, similar approaches were applied to dissociate strongly linked agronomic traits using ionising agents capable of inducing DNA breaks. For example, resistance to *Puccinia coronate avenae* and susceptibility to *Helminthosporium victorie* pathogens in oat have been separated by applying ionising radiation (Favret, 1960). Not only crops but also ornamental plants have been subjected to mutation breeding. In the Netherlands, a tulip variety with a flower colour mutant developed by X-rays irradiation of bulbs was released in 1949. More varieties were developed in the following years, when another tulip variety was released in 1954 and the carnation variety in 1962 (Ibrahim et al., 2018).

In the early 1960s, mutational breeding started to emerge as a powerful tool to induce genetic variation not only in sexually propagated plants but also in vegetatively propagated ones and data were accumulating on the application of physical mutagens to induce mutations in several economically important plants. For example, experiments describing the application of X-ray irradiation in potato, *Chrysanthemum* and flower bulbs started to accumulate, as well as new experiments demonstrating the applicability of physical mutagens in fruit trees which were irradiated using a source of ^{60}Co (Nybom, 1961).

In the attempt to coordinate the effort in mutation breeding among different countries and institutions, the Joint FAO/IAEA Centre of Nuclear Techniques in Food and Agriculture was established in 1964.[6] Since then, the number of mutated plant varieties steadily increased at least until the 1980s when new and more advanced technologies in molecular biology became available. To date, over 3,200 plant varieties which have been produced by mutagenesis approaches are registered in the FAO/IAEA database with more than half of the registered varieties coming from rice (*Oryza sativa*), barley (*H. vulgare*), *Chrysanthemum* spp., wheat (*Triticum* spp.), soybean (*Glycine max*) and maize (*Zea mays*) (Jankowicz-Cieslak and Till, 2016b; Jankowicz-Cieslak et al., 2016b; Spencer-Lopes et al., 2018; FAO/IAEA, 2020).

4.2. Addressing ToR1: to provide a more detailed description of random mutagenesis techniques as applied *in vivo* and *in vitro*

4.2.1. What random mutagenesis techniques are used to obtain mutant plants?

4.2.1.1. Mutation breeding: summary of the main steps

The application of physical and chemical mutagenesis techniques in mutation breeding consists mainly in three steps. Step 1 is the induction of random mutations in the plant genome, step 2 is the screening of the obtained mutants to identify desired phenotypes, and finally, step 3 is the selection for the desired characteristics (Jankowicz-Cieslak et al., 2016a). The different mutagenesis techniques applied in mutation breeding (Step 1) are described in more details in the sections below. Regarding step 2, the screening of the mutants obtained by such techniques can follow the 'forward' or the 'reverse' genetic approach (Lee et al., 2014). The forward genetics approach is phenotype-driven since it consists first in the direct evaluation of the phenotype of the mutagenised plants (e.g. chlorophyll and leaf abnormalities, stem height, number of seeds, etc.) followed by the identification of the mutation causing the phenotype which can be assisted by molecular methods, such as polymerase chain reaction (PCR)-based markers. On the contrary, the reverse genetics approach consists first in identifying the sequence-specific mutation and then to characterise the phenotype which is caused by such mutation. For example, the identification of the sequence-specific mutation can be achieved by exploiting the DNA heteroduplex which forms between the wild type and the mutated DNA fragment in a procedure called Targeting Induced Local Lesions in Genomes (TILLING) (Tadele et al., 2010; Till

[6] http://www-naweb.iaea.org/nafa/about-nafa/Joint-FAO-IAEA-History.html

et al., 2012; Lee et al., 2014). Finally, after mutation breeding step 3, the plant carrying the desired mutation enters a complex selection programme aimed at incorporating such mutation(s) in the cultivated/commercial plant varieties and removing other unwanted mutations (Spencer-Lopes et al., 2018).

4.2.1.2. General considerations on in vivo and in vitro random mutagenesis techniques in plants

Random mutagenesis techniques, including physical (Section 4.2.1.3) and chemical (Section 4.2.1.4) mutagenesis, can be applied to a wide range of plant material. These techniques can be used to treat plant material like whole plant, seeds, tuber, rhizomes, stems, buds, bulb, pollen, leaf and stem explants, anther, embryos, microspores, callus, cell cultures and other plant propagules (Suprasanna et al., 2014; Bado et al., 2015). Seeds are the material of choice in sexually propagated species because they are easy to handle in large quantities, easy to transport and to store, especially after treatment (Bado et al., 2015). According to the definition provided in the background of this document (Section 1.1), *in vitro* treatment of plant cells would be followed by the regeneration of the whole plant.

The application of random mutagenesis techniques to plant material *in vitro* offers some advantages compared to the application *in vivo*, such as the uniformity of the treatment and the possibility to apply a selective agent more easily, to screen large populations in a relatively small space and to handle disease-free plant material (Suprasanna et al., 2012). Moreover, somaclonal variation, which is defined as the genetic diversity observed in progeny of plants regenerated from tissue cultures, can also be exploited to increase genetic diversity in the cell population. Somaclonal variation can be coupled with random mutagenesis techniques when applied *in vitro* to further increase the mutation frequency (Suprasanna et al., 2014). Compared to *in vivo* mutagenesis, random mutagenesis performed *in vitro* on plant cell cultures may facilitate the selection of some agronomic traits such as for example tolerance to herbicide, salts, metal, flooding, cold and drought, or the selection of the embryo- gametophyte-lethal mutations for asexually reproducible plants (Suprasanna et al., 2014).

Regardless of the method used in random mutagenesis, chimerism can affect the plant obtained from the mutagenised plant tissue. Chimerism is defined as 'sectoral differences in a mutant, whereby cells of different genotypes exist side-by-side in the tissues of the same individual plant' (Mba, 2013). Both sexually and asexually (vegetatively) propagated plants regenerated from material which were treated with either chemical or physical mutagens or a combination of both would display chimerism. However, chimerism can be eliminated in sexually propagated crops by selfing or crossing. This cannot be achieved in vegetatively propagated crops where the genetic chimerism state of the tissue remains in the genetic background in the following propagation steps. However, techniques have been developed to deal with this limitation and to improve the genetic homogeneity of the treated material. Indeed, chimerism can be addressed using *in vitro* random mutagenesis when the whole plant is regenerated starting from a single mutagenised cell, thus exploiting the totipotency characteristic of plant cells. For example, this approach is used in banana and *Chrysanthemum* (Datta and Chakrabarty, 2009; Christov et al., 2014; Jankowicz-Cieslak and Till, 2016a). In this case, chimerism can be eliminated.

The heterozygosity of the mutated loci is another important aspect in random mutagenesis (Mba, 2013). Indeed, most of the generated mutations are in heterozygous state (i.e. for diploid organisms, only one copy of the two alleles is mutated). This could represent a problem when the phenotype is manifested only when the mutation is in homozygous state. This limitation can be overcome by selfing in case of sexually propagated crops. Also, the induction of doubled haploids (DH), when the whole plant is regenerated from the doubling of the chromosome number of gametic cells, pollen and egg cells, can be exploited to address the problem of heterozygosity of the mutated loci at least in sexually propagated plants (Mba, 2013). The possibility to combine DH systems with mutagenesis techniques may represent an advantage that can save time and effort in selecting pure breeding lines containing the mutation in homozygous stage (Maluszynski et al., 2009). However, the use of DH lines is limited to those plant species for which such method has been established, like the manipulation of anther and microspore *in vitro*, the use of wide hybridisation, and the ovary and ovule's cultures (Maluszynski et al., 2009). In recent years, researchers have developed haploid mutagenesis for many plant species including wheat, triticale, maize, *Brassica napus*, liverwort and tobacco (Shen et al., 2015). However, the recessive nature of the mutations and the heterozygosity of the mutated loci still represent a problem for vegetatively propagated plants.

4.2.1.3. Physical mutagenesis techniques applied in mutation breeding

Physical mutagenesis consists in the application of physical mutagens including ionising and non-ionising radiation to generate mutations in plant material. The exposure to the physical mutagen can be chronic, acute or fractionated. In chronic exposure, the plant material is exposed to a relatively low dose of radiation for a long period of time (weeks or months). Conversely, in acute exposure, the plant material is exposed to a high dose of radiation for a short period of time (seconds or minutes) (Mba et al., 2010). The fractionated approach consists of irradiating plant material at discrete time intervals to allow the material to recover between treatments (Kodym et al., 2012; Bado et al., 2015). Table 2 summarises the physical agents which are commonly used for random mutagenesis in plants.

X- and γ-rays are the most used ionising radiation in random mutagenesis. The source of radiation used for γ-rays is ^{60}Co or ^{135}Cs and to a less extent ^{239}Pu which can be used in contained equipment in laboratory conditions (i.e. gamma cell irradiators) or in the field (i.e. gamma chamber or room, field, or greenhouse) (Mba, 2013; Riaz and Gul, 2015). For the non-ionising radiation, irradiation with UV rays is the most used method. However, the use of UV treatment in random mutagenesis is more limited compared to X- and γ-rays because UV rays can penetrate plant material to a lesser extent compared to the other ionising radiations (Bado et al., 2015). Within the UV radiation spectrum, UV-C rays are the most frequently used to treat cells (Riaz and Gul, 2015; Spencer-Lopes et al., 2018). Fast neutron bombardment (FNB) has also become a popular physical mutagen. FNB usually causes larger chromosomal deletions, loss of chromosomes and translocation in the plant genome compared to γ-rays which usually cause smaller deletions (Riaz and Gul, 2015). Ion beams, mainly produced by particle accelerators using ^{20}Ne, ^{14}N, ^{12}C, ^{7}Li, ^{40}Ar or ^{56}Fe as radioisotope sources, started to gain traction in the early 1990s in China and Japan. The spectra of mutations produced by ion beams is supposed to be distinct from those caused by X- and γ-rays because the first ones possess a high linear energy transfer compared to the latter ones (Magori et al., 2010; Mba, 2013; Suprasanna et al., 2014). Alpha particles, which are analogous to helium nucleus, have a more limited penetrating capability compared to β-particles which are produced by ^{3}H, ^{32}P and ^{35}S. Irradiation with β-particles can be achieved by the administration of ^{32}P and ^{35}S to the growing plants followed by the incorporation of these radioactive isotopes into the cell nuclei. However, the irradiation dose from β-particle is difficult to estimate compared to X- and γ-radiation and hence its application has been more limited (Mba, 2013; Spencer-Lopes et al., 2018). Cosmic radiation has also been used to mutagenise plants but only under vacuum or under microgravity conditions in the outer space. Although not easily accessible, this approach was used to generate tens of commercial varieties in rice, wheat, cotton, sesame, pepper, tomato, sesame and alfalfa (Tanaka and Hase, 2010; Suprasanna et al., 2014).

The choice of plant material to be irradiated varies depending on the plant species to be mutagenised. Seeds are commonly used for seed-propagated crops (e.g. rice) while plants propagules/explants (e.g. carrying meristematic tissues) are necessary in case of vegetatively propagated crops (e.g. cassava) (Mba et al., 2010) (see Section 4.2.1.2). The optimal dose of radiation depends on the species and the genotype considered (Lee et al., 2014). In physical mutagenesis, the absorbed dose is currently measured in gray (Gy) which corresponds to 1 Joule per kg of treated material (1 Gy = 1 J kg^{-1}) (Spencer-Lopes et al., 2018). A general rule is to apply an irradiation dose which results in a lethal dose of 50% of treated material (LD_{50}) or reduction of 30–60% in growth ($GR_{30\text{-}60}$) of the treatment material in M1 (Mba, 2013; Bado et al., 2015). Dose optimisation can be achieved by assessing some plant performance indicators such as germination percentage, seedling survival, chlorophyll abnormality incidence, growth rates and relative sizes of specific parts of the plant (Lee et al., 2014). In general, radiosensitivity (i.e. the susceptibility of the treated material to ionising radiation) can be estimated by measuring several parameters like the reduction in seed germination or height of the seedlings, the level chromosomal aberrations, the height, width and number of buds or leaves, the sterility of M1 plants, and the frequency of induced mutations (Datta, 2014). Radiosensitivity depends also on the species genotype (ploidy), seed size, the stage of cellular development, the age of the irradiated tissue, the storage methods after irradiation, the oxygen level, the presence of other chemicals, the temperature and the water content. Water content, in particular, is one of the most important aspect affecting the outcome in physical mutagenesis (Datta, 2014). Radiosensitivity is also dependent on interphase chromosome volume (ICV) which is 'the defined as the nuclear volume divided by the chromosome number, in DNA content, and ploidy level'; a high ICV value usually correlates with an increased sensitivity to physical mutagens (Kodym et al., 2012).

4.2.1.4. Chemical mutagenesis techniques applied in mutation breeding

Chemical mutagenesis consists in the application of chemical agents to generate mutations in the plant genome. The list of chemical mutagens includes, among others, alkylating agents, intercalating agents, and base analogues (Mba et al., 2010). Table 3 summarises the chemical agents which are commonly used for random mutagenesis in plants.

The most used chemical mutagens in mutation breeding are ethyl methanesulfonate (EMS), methylnitrosourea (MNU), 1-ethyl-1-nitrosourea (ENU) and sodium azide (SA). MNU and SA are often used in combination (Leitao, 2012; Riaz and Gul, 2015). As for physical mutagens, also for chemical mutagens the right dose should be determined. The dose is usually equivalent to the concentration of the chemical per the duration of the treatment (Spencer-Lopes et al., 2018). The chemical's concentration and time of exposure varies depending on the chemical itself and the plant material/ plant species considered. The evaluation of LD_{50} at M1 generation can be considered the best proxy to quantify the amount of mutagen to be used (Lee et al., 2014). Also, temperature (usually in the range 20–25°C) is an important parameter to consider in chemical mutagenesis (Mba et al., 2010; Spencer-Lopes et al., 2018). The pH of the solution used to dissolve the chemical mutagen should be neutral or slightly acidic (pH 6–7) to reduce the decomposition of the mutagen itself. Overall, the chemical concentration, the time of exposure and the pH has anyway to be adjusted depending on the chemical and the plant tissue to be treated (Leitao, 2012).

Different plant materials can be used in chemical mutagenesis, ranging from whole plant to *in vitro* cell cultures. Seeds represent the most used plant material for chemical mutagenesis (Leitao, 2012). However, the *in vitro* application of mutagens to explants became more common in recent years providing some useful tools for mutagenesis in vegetatively propagated plants, especially to resolve chimerism (Jankowicz-Cieslak and Till, 2016a) (see section 4.2.1.2). The plant material should preferably be in the active growing stage. Woody material or plant material characterised by thick layers may be recalcitrant to the chemical treatment since the mutagen might not efficiently penetrate the tissue (Spencer-Lopes et al., 2018). Indeed, depending on the plant material used (seeds, plant propagules, buds, etc.), the chemical mutagen must reach the apical and/or the axillary meristems to induce the mutations to be passed to the next generation. Also, chemical mutagens might be affected by degradation during the incubation time. In this case to avoid reduction in the efficacy, the renewal of the soaking solution might be needed (Leitao, 2012).

Table 2: List of common physical agents used in physical mutagenesis (Maluszynski et al., 2009, 2016; Magori et al., 2010; Leitao, 2012; Bado et al., 2015; Suprasanna et al., 2015; Spencer-Lopes et al., 2018)

Physical agent	Abbreviation	Type	Source
γ-rays	–	Ionising	^{60}Co – ^{137}Cs
X-rays	–	Ionising	X-ray tubes with cylindrical anode
UV light	–	Non-ionising	UV lamps
Protons	–	Ionising	Particle accelerators Cosmic radiation
Fast neutron bombardment	FNB	Ionising	Nuclear reactors/atomic piles
α-particles	–	Ionising	Radionuclide ^{32}P
β-particles	–	Ionising	Radioactive isotopes ^{7}Be
Ion beam radiation	IBR	Ionising	^{20}Ne, ^{14}N, ^{12}C, ^{7}Li, ^{40}Ar, He, H, Si or ^{56}Fe as radioisotope sources accelerated by cyclotron/synchrotron

Table 3: List of common chemical agents used in chemical mutagenesis (Maluszynski et al., 2009, 2016; Magori et al., 2010; Leitao, 2012; Bado et al., 2015; Suprasanna et al., 2015; Spencer-Lopes et al., 2018)

Chemical mutagenesis		
Chemical	**Abbreviation**	**Type**
Ethyl methanesulfonate	EMS	Alkylating
N-ethyl-*N*-nitrosourea	ENU (ENH)	Alkylating
N-methyl-*N*-nitrosourea	MNU (MNH)	Alkylating
Ethyleneimine	EI	Alkylating
Diethyl sulfate	DES	Alkylating
N-methyl-*N1*-nitro-*N*-nitrosoguanidine	MNNG	Alkylating
1-ethyl-2-nitro-1-nitrosoguanidine	ENNG	Alkylating
Dimethyl sulfate	DMS	Alkylating
Methyl methanesulfonate	MMS	Alkylating
N,*N*-diethylnitrous amide	NDEA	Alkylating
N,*N*-dimethylnitrous amide	NDMA	Alkylating
Diepoxybutane	DEB	Alkylating
Sodium azide	SA	Mutagenesis mediated by organic intermediate (L-azido-alanine)
Base analogues (e.g. 5-bromo-uracil, 5-bromo-deoxyuridine)	–	Base analogues
Intercalating agents (e.g. ethidium bromide, acridines)	–	DNA intercalating agent

4.2.1.5. Examples of in vivo and in vitro random mutagenesis applications in plants

Thousands of plant varieties with modified agronomic traits have been obtained in mutation breeding in the 20th century. Most of the mutant cultivars have been generated by physical mutagenesis because physical mutagens were the first to be studied and applied in mutation breeding. However, chemical mutagenesis has been extensively used in more recent years. In this chapter, some examples extracted from the outcome of the literature search are described.

Important staple crops varieties have been developed using mutation breeding programs. Rice (*O. sativa*) mutants were created by both physical and chemical mutagenesis during the last decades. For example, *in vitro* chemical mutagenesis was used to treat rice anthers with EMS, rice varieties with increase amylase activity were generated by SA application, and rice calli treatment with EI were used to isolate plant mutants with enhanced rice blast resistance (Ryu et al., 2003; Riaz and Gul, 2015). The application of physical mutagenesis such as γ-rays in the range 50–350 Gy, ion beam radiation using carbon and neon in the range of 20–50 Gy, and fast neutron bombardment at 20 Gy allowed the selection of rice mutants in plant development and metabolism, in industrial and nutritional quality, in biotic and abiotic stress tolerance, and in herbicide tolerance (Mba et al., 2010; Viana et al., 2019). Interestingly, *in vivo* random mutagenesis of rice seeds was also performed with a combination of physical (γ-rays) and chemical (EI) mutagens to increase the percentage of chlorophyll mutants in rice (Ando, 1968; Riaz and Gul, 2015). In Japan, tens of rice mutant varieties have been registered since the first semi-dwarf mutant rice cultivar 'Reimei', developed by gamma radiation, was released in 1968. Other important rice mutant varieties include the 'LGC-1' rice, developed by chemical mutagenesis (EI), which contains low amount of digestible glutenin and high amount of indigestible prolamine. This rice variety is useful for patients in need of a low protein diet (Nakagawa, 2009). Other rice mutants, including leaf colour, low phytic acid, giant embryo, and high resistant starch mutants, have also been developed through the application of physical mutagens (mainly γ-rays and ion beams) sometimes in combination with *in vitro* culture techniques. Some of these rice mutants have been used in hybridisation programs with elite rice varieties (Amano, 2006).

Barley (*H. vulgare*) has been not only one of the first crops to be subjected to agronomic traits improvement by mutation breeding, but it has also been used as model crop to test the effect of physical and chemical mutagens since the beginning of the 20th century. For example, barley seeds

have been subjected to *in vivo* random mutagenesis to compare the effect of physical (γ-rays and FNB) and chemical (EMS) mutagens to induce chlorophyll-deficient mutants (Constantin, 1975). The author concluded that the difference observed may be related to the recovery and detection rather than the process of induction of mutations. In barley, the use of physical mutagens such as X-rays contributed to the development and selection of mutant varieties with important agronomic traits, for example dense spikes (cultivar 'Pallas' released in 1958) and early maturity mutants (cultivar 'Mari' released in 1960) (Lundqvist, 2009).

Lettuce (*Lactuca sativa*), which is one of the most consumed fresh vegetable, has been also subjected to *in vivo* random mutagenesis. Lettuce seeds were treated with chemical mutagen (EMS) to generate an early flowering and dwarf double-mutant which was crossed with cultivar 'Salinas'. The resulting cultivars 'Ice Cube', 'Blush' and 'Mini-Green' are now available on the market. Lettuce seeds were also mutagenised using physical agents (γ-rays and FNB) to identify genes involved in powdery mildew disease resistance (Mou, 2011). Seed from sweet pepper (*Capsicum annuum*) cultivars were also mutagenised using chemical (EMS) and physical (γ-rays) mutagens to obtain varieties with determinate habit, lack of anthocyanins, changes in fruit shape and position, and to develop hybrid with increased β-carotene content (Tomlekova et al., 2009).

Although under-represented among the crops subjected to random mutagenesis, vegetatively propagated crops have also been subjected to mutation breeding programmes. Sugarcane (*Saccharum* spp.), for example, is a difficult species to breed and developing new varieties with enhanced traits may take up to 12–15 years. For this reason, *in vitro* random mutagenesis using both chemical (EMS) and physical (γ-rays) agents applied to micro-propagation of meristematic tissue and tissue cell cultures have proved to be successful. *In vitro* mutagenesis of sugarcane allowed the development of varieties with enhanced traits such as smut resistance, salt tolerance, improved agronomic traits like sugar content, number of millable canes, girth and yield (Suprasanna et al., 2011; Devarumath et al., 2015). Potato (*Solanum tuberosum*) is also a vegetatively propagated crop due to its genetics and biology which limit cross breeding programs. *In vitro* random mutagenesis of potato helped creating salt-tolerant varieties using γ-rays (Kiunga et al., 2014).

Pulses were also subjected to chemical and physical mutagenesis to improve important agronomic traits. For example, seeds of mung beans (*Vigna radiata*) were treated with chemical (EMS and SA) and physical (γ-rays) agents to obtain mutants in key agronomic traits such as plant height, days to flowering and maturity, seed yield and leaf morphology. (Auti and Apparao, 2009; Wani et al., 2014). Chickpea (*Cicer arietinum*), a self-pollinated crop with very limited sexual compatibility with most wild genotypes, was also treated with different physical and chemical mutagens to improve its genetic makeup and to select mutants in chlorophyll content and plant morphology, in seed protein and mineral content, and in low levels of anti-nutritional factors (Rafiq Wani et al., 2014). FNB was used in the legume model *Medicago truncatula* to create over 1,50,000 mutant lines to study key aspects in the legume family such as symbiotic nitrogen fixation and nodule development (Chen and Chen, 2018). Soybean (*G. max*) has been subjected to random mutagenesis using EMS and X-rays to produce population of mutants to be screened using TILLING approach that help identifying genes involved in control of maturity and fatty acid metabolism (Anai, 2011).

Random mutagenesis has also been applied to agronomically important fruit trees such as *Citrus* spp., a perennial plant which has a long reproductive cycle, and which is mainly vegetatively propagated. Different *Citrus* spp. plant materials (seeds, buds, calli, protoplasts, decapitated young seedlings, epicotyl segments, micrografted shoot tips) have been subjected to both *in vivo* and *in vitro* random mutagenesis using mostly physical mutagens (X- and γ–rays, and thermal neutrons). The obtained agronomic traits are mainly related to the reduced number of fruit seeds, resistance to diseases, improved fruit colour, alteration in fruit harvest time and plant height (Latado et al., 2012).

In vitro random mutagenesis has been extensively used also in ornamental plants such as Orchidaceae*. In vitro* cultured protocorm-like bodies were exposed to different doses of gamma radiation depending on the different species (Sheela and Sheena, 2014). *In vivo* and *in vitro* random mutagenesis were also applied to other ornamental plants such as *Anthurium* using physical (γ-rays) and chemical (EMA) mutagens to treat seeds and plantlets (Sheela and Sheena, 2014). Large number of mutants of ornamental plants have been obtained by random mutagenesis, including for example mutants in flower colour and shape, and chlorophyll variegation in leaves in genus and species such as *Bougainvillea*, *Chrysanthemum*, *Gladiolus*, *Hibiscus rosa-sinensis* cv. Alipur Beauty, *Perennial portulaca*, *Polianthus tuberosus*, rose, *Lantana depressa* and many others (Banerji, 2014; Christov et al., 2014).

Plant species which are an important source for biofuel production have been subjected to mutation breeding. For example, *Jatropha curcas* has been mutagenised both *in vivo* and *in vitro* using

both physical (X- or γ-rays) and chemical (EMS) mutagens to treat seeds and stem cuttings (Maghuly et al., 2016). Different *J. curcas* mutants were obtained by random mutagenesis techniques including both tall and dwarf, high branching, high fruit and oil yielding, and high biomass yielding variants (Christov et al., 2014). Chemical and physical mutagenesis have also been used to generate plants to be used in phytoremediation. Examples include *Brassica juncea* tolerant to lead, *H. vulgare* tolerant to aluminium, *Pisum sativum* tolerant to cadmium and *Helianthus annus* L. tolerant to zinc and cadmium (Phang et al., 2012).

4.2.2. Are all these techniques applicable both *in vivo* and *in vitro*?

All these techniques are applicable both *in vivo* and *in vitro*. The information provided in Section 4.2.1 illustrates how random mutagenesis techniques, which includes chemical and physical mutagens listed in Tables 2 and 3, can all be applied to plant material both *in vivo* and *in vitro* settings although the dose, the exposure time, and the overall experimental protocol may vary. Moreover, several examples are also given in Section 4.2.1.5 to demonstrate that both chemical and physical mutagenesis techniques have been indeed used in different plant species both *in vivo* and *in vitro*.

4.3. Addressing ToR3: to assess whether the molecular mechanism underlying random mutagenesis techniques is different if the techniques are applied *in vivo* or *in vitro*

4.3.1. What are the underlying molecular mechanisms which generate the mutations?

All living organisms, including plants, have to deal with natural mutagens that may cause changes in their genomes by triggering DNA damage or alteration. When an alteration is detected by the cells repair machinery, the eukaryotic cells slow down or stop cell cycle at an available checkpoint to repair the damaged DNA. Repairing DNA damage or alterations is an important process to preserve the stability and transmission of genetic information to the next generations. This efficiency of the repair process varies, and can result in the full repair to the original DNA sequence (conservative repair), or be more error prone and (non-conservative) leading to repair with integration of DNA sequence changes, i.e. mutations. In this paragraph, we will first review the mechanisms by which the mutagens generate lesions (breaks) in the DNA and then the different cell repair mechanisms triggered by the lesion in a second section.

4.3.1.1. Mechanisms leading to DNA damage

DNA damage caused by physical agents

Ionising radiations include X-ray, γ-rays, neutrons and high-energy ion beams. Ionising radiations are the most commonly used physical mutagens (Mba, 2013; Navjot et al., 2018; Singer et al., 2021). Radiations are capable of dislodging electrons from the orbits of the atoms that they hit, transforming those atoms into ions. As a consequence of the ionisation process, free positive radicals and free electrons are produced. The free radicals in solution will recombine to form stable products (Spencer-Lopes et al., 2018). Ionising radiations can also cause DNA alterations indirectly, by radiolysis of the water molecules, which generates hydroxyl radicals (•OH). These radicals can attack the DNA bases and the sugar residues in various ways, creating base lesions. Moreover, free radicals generated by ionising radiations are also able to create damages to the phosphodiester bonds, inducing single strand breaks (SSBs) with 3′ phosphate or 3′ phosphoglycolate ends, rather than 3′ hydroxyl ends (Chatterjee and Walker, 2017). A series of SSBs in close proximity and on both strands could give rise to double stranded breaks (DSBs) (Barnard et al., 2013). As a result, gamma rays can cause small deletions of few nucleotides, but also larger deletions or inversions have been reported (Hernández-Muñoz et al., 2019; Viana et al., 2019).

Ion beams induce mutation at high frequency and have allowed the selection of a large spectrum of phenotypes (Viana et al., 2019). Compared to gamma rays, ion beams have a higher linear energy transfer (LTE), causing typically larger deletions, greater than 1 kb, and intergenic rearrangements like inversions, translocations and insertions (Viana et al., 2019).

UV light (250–290 nm) is less penetrating, compared to ionising radiation and, for this characteristic, it is mainly used to mutagenise single cells or mono-layer tissues, such as spores, suspension cell cultures and pollen grains (Navjot et al., 2018; Spencer-Lopes et al., 2018). There are

three classes of UV radiation, UV-C (190–290 nm), UV-B (290–320 nm), and UV-A (320–400 nm). UV light triggers the formation of covalent linkages between two pyrimidines. There are two major types of lesions caused by UV light: cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6–4) pyrimidone photoproducts [(6–4) PPs] (Manova and Gruszka, 2015). CPDs are characterised by having the C-5 and C-6 carbon atoms of two adjacent pyrimidines (usually TT) covalently bonded; in 6-4 PPs the two linked carbon positions are the C-6 and C-4 of a TC dinucleotide. The dimerisation causes a distortion in the geometry of the helix, often leading to a block of transcription (Manova and Gruszka, 2015) or a stall in replication that has to be overcome by specialised repair mechanisms (see below) (Chatterjee and Walker, 2017). These alterations can lead to single nucleotide changes, or to larger mutations that cause chromosomal rearrangements (Shu et al., 2012).

Fast neutron (FN) irradiation, or neutron bombardment, has also been utilised to mutagenise crops. FN mutagenesis generally results in deletions, and translocations. Fast Neutrons have been reported to cause higher number of non-repairable double lesions and a higher frequency of double stranded DNA breaks, compared to gamma rays, most probably due to higher ion density. Mutagenesis by Fast neutrons has been reported in several species. The reported lesions range from 1 bp to 18 Mb, with 1-4 kb deletions being the most frequent ones (Kumawat et al., 2019).

DNA damage caused by chemical agents

Despite the large number of chemical compounds that have mutagenic effects, only a few are routinely used in plant breeding.

Alkylating agents are mutagenic compounds with strong affinity for the bases and phosphodiesters of the DNA molecule. These compounds are able to transfer their alkyl group to DNA molecules that contain nucleophilic groups. There are two main groups of alkylating agents: compounds that belong to the first group produce a reactive intermediate, independently of the substrate (the DNA), and are able to transfer the alkyl group to the nitrogen, the oxygen, and phosphate group of the DNA molecule. The second group includes compounds that react directly with the most nucleophilic sites on DNA, like the nitrogens N7 and N3 of guanine and N3 of adenine.

Alkylating agents target preferentially 11 sites in the four bases and phosphodiester groups of the DNA molecule. Of these, O6 alkylguanine is strongly mutagenic since it causes a mismatch with thymine and gives rise to G:C – A:T transitions. EMS is the most commonly used agent that is able to alkylate the O6 position (Leitao, 2012). The characterisation of mutants has demonstrated that EMS has a sequence bias for guanine residues in the context R**G**CG, where R is A or G and the mutated G is in bold (Spencer-Lopes et al., 2018). O4 alkylthymine, a lesion generated by ENU, can mispair with guanine and lead to A:T – G:C transitions. MMS, another important alkylating agent, induces the formation of N3 alkylguanine and N3 alkyladenine, leading to transversions or transitions (Leitao, 2012; Singer et al., 2021).

Overall, alkylating agents are able to produce transitions, transversions, insertions, deletions, inversions, DNA single and double strand breaks, lesions that are similar to those caused by ionising radiations (Leitao, 2012).

Sodium azide (NaN3; SA) is an inorganic, highly toxic compound, inhibitor of cellular respiratory processes in living cells (Spencer-Lopes et al., 2018). Sodium azide is also considered a pro-mutagen, as it generates a reactive intermediate, which apparently does not interact directly with DNA, and the mutations are rather mediated by the host-plant cellular processes involved in DNA excision-repair. SA predominantly causes transitions from G:C to A:T, with a strong context bias for the sequence GGR (Viana et al., 2019).

4.3.1.2. Mechanisms leading to repair

The detection of DNA damage triggers cellular repair mechanisms than can lead to the restoration of the original sequence. However, the fidelity of the repair is not always complete (100%) and can lead to the introduction of DNA sequence change. If the sequence change is transmitted to the next generation, it will be a mutation. It is important to notice that the DNA damage and consecutive cellular repair mechanisms described below are identical, whether the damage is caused by an induced or by a spontaneous event.

Reversal of DNA damage

Some alterations are usually repaired with error-free pathways, which do not lead to mutations and are reported here just for completeness. One of them is the photoreactivation, based on photolyase

enzymes, utilised to repair UV-caused damages (Tuteja et al., 2009; Shu et al., 2012; Manova and Gruszka, 2015).

In mammals and bacteria, DNA damages caused by alkylating agents are dealt with by alkyltransferarase enzymes (AGT), which transfer the alkyl group from the alkylated oxygen of the DNA base to a cysteine in their active site. Plants lack AGT orthologs and they are likely to have developed alternative mechanisms to repair alkylated bases (Shu et al., 2012; Manova and Gruszka, 2015), like mechanisms based on excision repair (see below).

Excision repair mechanisms

Base excision repair (BER) mechanisms repair alkylation and other kinds of damages that do not cause helix distortion. DNA glycosylases first cause a bend in the helix in order to flip the targeted base in their catalytic site. They cleave the N-glycosidic bond and remove the base residue from the site. The repair progresses in subsequent steps, where the a-basic site is deprived of the 5′ terminal deoxyribose-phosphate residue, and the gap is filled with the help of by polymerases and ligases (Shu et al., 2012).

Bulky lesions that cause transcription blocks or distortion in the double helix, such as CPDs, 6-4PPs, or cross-links between alkylated complementary bases, are usually repaired by the nucleotide excision repair (NER) pathway (Shu et al., 2012). Two sub-pathways have been identified, the global genome repair (GGR) and the transcription coupled repair (TCR), which differ in the enzymes involved in the initial recognition of the lesion. NER typically removes a stretch of 20–30 nucleotides that contain the lesion and use the native strand as a template to copy and fill the gap. An important enzyme involved in the process is TFIIH, also a component of the RNA polymerases I–III holoenzymes, which plays a primary role in unwinding the double helix. The process continues with specific endonucleases that produce the nick, and the gap is filled by replication factors and DNA polymerase (Tuteja et al., 2009). Genes involved in the NER pathway have been shown to be active in photoreactivation and homologous recombination (HR) (described below).

Post replication repair

DNA damage can have different consequences on cell functions. Alterations that distort the geometry of the double helix can cause a stall in the replication process. A lesion affecting the leading strand may stall the DNA replication fork, causing an accumulation of ssDNA, which can lead to DNA breaks. Even if the replication starts from a distal origin of replication, this would create gaps. Post replication repair (PRR) mechanisms do not repair the lesions, but they act to bypass them, in an attempt to fill the gaps and allow the replication to continue. A well-studied mechanism is the trans lesion synthesis (TLS), based on TLS polymerases. The process works in two subsequent steps: when a lesion is detected, TLS polymerases replace the replication polymerases and add a nucleotide opposite the lesion. In a second step, another TLS polymerase inserts other nucleotides to extend the primer beyond the lesion (Shu et al., 2012; Sakamoto, 2019). Several TLS polymerases have been identified in plants. They all lack the 3′-5′ proofreading activity, which makes them more prone to incorporate the unprimed nucleotides. Plants with mutated POLη, POLζ, REV1 polymerases have been shown to be sensitive to UV radiation, which demonstrates how these polymerases are needed to by-pass the effects of UV radiation, promoting normal tissue development. At the same time, though, their low fidelity is often the cause of higher mutation rates (Sakamoto, 2019).

Mismatch repair (MMR)

As described above, mismatches deriving from replication of damaged DNA are more frequent than those occurring spontaneously during replication of undamaged DNA. Mismatch Repair (MMR) is a conserved PRR pathway, used to repair mismatches deriving from replication and loops generated by insertion/deletions. MSH (MutS homolog) enzymes form dimers involved in the recognition of the lesion. Exonucleases hydrolyse the phosphodiester bond at the mismatch and a POL δ adds the correct nucleotide, followed by ligases to seal the gap. MMR is responsible to reduce mismatch rates up to 100-fold (Sakamoto, 2019).

DNA Double Strand Break repairs

Lesions that cause breaks on both DNA strands – DNA DSBs - are particularly important, as they can potentially lead to loss of genetic material, if not repaired properly. Physical mutagens, like ionising or UV Radiation can cause DSBs. Also, mechanisms involved in the repair of lesions caused by chemical agents, like BER or NER, form single stand DNA break (SSB) intermediates, which could

eventually lead to the formation of double strand breaks. Cells have evolved mechanisms to detect and repair DSBs. Plants use protein kinases to communicate the presence of a break. Ataxia telangiectasia mutated (ATM) kinase is the primary transducer for DSBs, whereas the kinase named Ataxia telangiectasia and RAD3-related (ATR) is involved in the transduction signal for the presence of SSBs. Upon recognition of a DSB, kinase proteins are activated that trigger a signal transduction pathway, which leads to a cell cycle arrest (Manova and Gruszka, 2015). Four mechanisms can be involved in repairing DSBs: the classical NHEJ (cNHEJ), the alternative NHEJ (aNHEJ), the single-strand annealing (SSA) and HR (Ceccaldi et al., 2016). If with cNHEJ the DSB is repaired independently of sequence homology, the three other repair pathways require the presence of homologous sequences in order to repair the damage.

cNHEJ can occur throughout the cell cycle but it is dominant in G0/G1 and G2. In this mechanism, that involves different factors such as Ku70/80, DNA-PKcs, and DNA ligase IV, the DSB is repaired by blunt end ligation and requires no or minimal homology (up to 4 bp). cNHEJ is conservative but adaptable, and the accuracy of the repair is dictated by the structure of the DNA ends. As a consequence, in some case, the processing of chemically incompatible DNA ends, that cannot be ligated directly, leads to small insertions or deletions.

Alternatively, the DSB end can be resected, leaving 3′ single-stranded DNA (ssDNA) overhangs. Depending on the extension of the resection, the DSB can be repaired by three possible mechanisms: HR, SSA and aNHEJ.

HR is active during S and G2 mitotic phases where the amount of DNA replication is highest and when the sister template is available. RAD51-mediated strand invasion by the 3′ ssDNA overhangs into the homologous sequence of the sister or homologous chromatid is followed by DNA synthesis at the invading end. This is considered as a conservative pathway and it does not lead to genetic loss. It is the typical mechanisms involved in crossing over events during meiosis (Viana et al., 2019).

The resected DSB can also be repaired by mutagenic repair pathways, namely SSA or aNHEJ. To be repaired by SSA, the DSB needs to be flanked by two repeated sequences oriented in the same direction. In this mechanism, after both strands of the same DNA duplex have been cut around the site of the DSB, the resulting 3 ‘overhangs align, allowing restoration of the continuity of the DNA molecule. Repair through SSA will end-up by the loss of the DNA sequence between the repeats and of one of the two repeats.

In the less characterised aNHEJ mechanism, the repair of the DSB involves annealing of micro-homologies (4–25 bp) before joining. It relies on the activity of DNA polymerase theta that promotes annealing of ssDNA containing micro-homologies and completes DNA synthesis to fill in the resected gap, before ligation terminates the repair. This mechanism is associated with excessive deletions and insertions at junction sites and has been implicated with the formation of large-scale genome rearrangements, including chromosomal translocations. The fact that aNHEJ serves as a backup for both cNHEJ and HR indicates that it might be active throughout the cell cycle.

4.3.2. Is there any difference between these molecular mechanisms whether they happen *in vivo* or *in vitro*?

There is no difference between these molecular mechanisms whether they happen *in vivo* or *in vitro*. As mentioned in Section 4.3.1.1, both physical and chemical mutagens cause alteration at the DNA level. These processes and the repair mechanisms that are triggered by the mutagens are acting at the cellular level and therefore are the same irrespective if the cell is part of a cultivated tissue *in vitro* or an organ of a plant *in vivo*.

4.4. Addressing ToR2: to assess whether the types of genetic modification induced by random mutagenesis techniques are different depending on whether the technique is applied *in vivo* or *in vitro*

4.4.1. What type of alterations at the DNA level are induced by random mutagenesis?

Spontaneous or induced mutagens lead to alterations of the DNA that, in spite of the cell repair mechanisms, lead in some cases to mutations.

4.4.1.1. Types of mutations

Mutations can be broadly divided into (a) localised changes to the original sequence of DNA and (b) mutations that have an overall impact on the structure of chromosomes.

At the nucleotide level, mutations can be distinguished in deletion, insertion, or substitution. A substitution corresponds to the replacement of one nucleotide by a different one. When a purine base is replaced by another purine (G by A or A by G), or a pyrimidine is replaced by another pyrimidine (C by T or T by C), the mutation is called transition. When a purine is replaced by a pyrimidine, or vice versa, the substitution is called transversion. Transitions, and in particular C to T, are the most common types of mutations (Pathirana, 2011).

The effect of a substitution within the coding sequence of a gene can lead to three different scenarios: (1) the change of the amino acid (missense mutation) which can have no, small or strong effect on the encoded protein depending on the physico-chemical properties of the new amino acid, (2) the formation of a stop codon which will lead to a truncated protein (non-sense mutation), or (3) the lack of any effect (silent mutation) when the substitution does not lead to any change in the resulting amino acid due to the degeneracy of the genetic code (Pathirana, 2011; Bado et al., 2015).

Insertions and deletions occur when one or more nucleotides are inserted into or deleted from the original DNA sequence, respectively. When insertions/deletions (indels) occur within a coding sequence, they may result in the shift of the reading frame, with a consequence in the protein synthesis, as the amino acid sequence of the newly formed protein becomes different from the wild type (Mba, 2013).

Repair of a DSB can also lead to chromosomal rearrangements (duplication, inversion, or translocation). These structural changes can be accompanied by insertions or deletions. (Pathirana, 2011). These changes can be intrachromosomal or involve different chromosomes.

4.4.2. Is there any difference between the mutations whether they are obtained *in vivo* or *in vitro*?

There is no difference between mutations obtained *in vivo* and mutations obtained *in vitro*. Because mutations are the final results of molecular mechanisms that cause alterations to the DNA and repair mechanisms that are the same *in vivo* and *in vitro*, as summarised in Section 4.3, the types of mutations are also expected to be the same. This is supported by the information provided in Sections 4.4.1 and 4.2.1.5, which show that the types of mutations obtained by *in vivo* and *in vitro* mutagenesis are the same.

4.5. Addressing ToR4: to assess whether *in vitro* random mutagenesis techniques are to be considered as different techniques compared to *in vivo* random mutagenesis techniques or on the contrary, if they are to be considered as a *continuum*

4.5.1. Are *in vitro* and *in vivo* random mutagenesis techniques considered to be different or not?

Random mutagenesis techniques (Section 4.2) are all applicable to both *in vitro* and *in vivo* conditions. Within *in vivo* and *in vitro* random mutagenesis methods, a large variation of experimental protocols are available, depending on the plant species and its biology, the material used to induce mutations, the dose and exposure time needed to achieve a desired outcome, the selection process, etc.

As described in Sections 4.3 and 4.4, the molecular mechanisms present in the cell and underlying the genetic mutation generated by a particular technique do not differ whether such technique is applied to plant material either *in vivo* or *in vitro*. Therefore, the outcome of the random mutagenesis will not differ, and the same type of mutations can be achieved with both *in vivo* and *in vitro* approaches. Therefore, *in vivo* and *in vitro* random mutagenesis techniques should not be considered different.

5. Conclusions

The EFSA GMO Panel was asked (1) to provide a more detailed description of random mutagenesis techniques as applied *in vivo* and *in vitro*, (2) to assess whether the types of genetic modification

induced by random mutagenesis techniques are different depending on whether the technique is applied *in vivo* or *in vitro*, (3) to assess whether the molecular mechanism underlying formation of new mutations induced by random mutagenesis techniques is different if the techniques are applied *in vivo* or *in vitro*, and (4) to assess whether *in vitro* random mutagenesis techniques are to be considered as different techniques compared to *in vivo* random mutagenesis or on the contrary, if they are to be considered as a continuum.

The GMO Panel concluded that random mutagenesis techniques can all be applied to plant material both *in vivo* and *in vitro* although the mutagen dose, the exposure time, and the overall experimental protocol may vary. The molecular mechanisms underlying the random induced mutagenesis techniques are the same as the ones involved in the formation of spontaneous mutations and they include: (a) mechanisms that lead to the DNA alterations, which are triggered by mutagens, and (b) mechanisms that lead to the detection and repair of the alteration, which depend on several factors, such as the cell type, the stage of the cell in the cell cycle, and the nature of damage. These processes and the repair mechanisms that are triggered by the mutagens act at the cellular level and are therefore the same irrespective if the cell is part of an isolated cell or cultivated tissue *in vitro* or any part of a plant *in vivo*. Because both the molecular mechanisms that cause the DNA damage and the repair mechanisms are the same *in vivo* and *in vitro*, the types of mutations are also expected to be the same.

Finally, the GMO Panel concluded that the distinction between plants obtained by *in vivo* and *in vitro* approaches is, therefore, not justified. Indeed, the same mutation and the derived trait can be potentially obtained using both *in vivo* and *in vitro* random mutagenesis and the resulting mutants would be indistinguishable.

References

Amano E, 2006. Use of induced mutants in rice breeding in Japan. Plant Mutation Reports, 1, 21–24.

Anai T, 2011. Potential of a mutant-based reverse genetic approach for functional genomics and molecular breeding in soybean. Breeding Science, 61, 462–467. https://doi.org/10.1270/jsbbs.61.462

Ando A, 1968. Mutation induction in rice by radiation combined with chemical protectants and mutagens. Vienna.

Auti SG and Apparao BJ (Shu QY), 2009. Induced mutagenesis in mungbean (*Vigna radiata* (L.) Wilczek). Vienna, Austria, Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, International Atomic Energy Agency.

Bado S, Forster BP, Nielen S, Ali AM, Lagoda PJL, Till BJ and Laimer M, 2015. Plant mutation breeding: current progress and future assessment. Plant Breeding Reviews, Oxford, UK, Wiley-Blackwell.

Bairu MW, Aremu AO and Van Staden J, 2011. Somaclonal variation in plants: causes and detection methods. Plant Growth Regulation, 63, 147–173. https://doi.org/10.1007/s10725-010-9554-x

Banerji BK (Tomlekova NB, Kozgar MI and Wani MR), 2014. Mutation breeding and mutants of ornamental plants: The role of NBRI for economic gains. Wageningen Acad Publ, Wageningen.

Barnard S, Bouffler S and Rothkamm K, 2013. The shape of the radiation dose response for DNA double-strand break induction and repair. Genome Integrity, 4, 1. https://doi.org/10.1186/2041-9414-4-1

Ceccaldi R, Rondinelli B and D'Andrea AD, 2016. Repair pathway choices and consequences at the double-strand break. Trends in Cell Biology, 26, 52–64. https://doi.org/10.1016/j.tcb.2015.07.009

Chatterjee N and Walker GC, 2017. Mechanisms of DNA damage, repair, and mutagenesis. Environmental and Molecular Mutagenesis, 58, 235–263. https://doi.org/10.1002/em.22087

Chen Y and Chen R, 2018. Physical mutagenesis in Medicago truncatula using fast neutron bombardment (FNB) for symbiosis and developmental biology studies. Methods in Molecular Biology. Place Humana Press Inc., Humana Press Inc. pp. 61–69.

Christov NK, Ilchovska D and Hristov KN (Tomlekova NB, Kozgar MI and Wani MR), 2014. Chemical mutagenesis, mutation breeding and quantitative genetic analyses of maize mutants: from theory to practice. Wageningen Acad Pub, Wageningen.

Constantin MJ, 1975. Mutations for chlorophyll deficiency in barley: comparative effects of physical and chemical mutagens. Third International Barley Genetics Symposium, July 7–12, 1975. Authors A-G.: [Abstracts].15

Datta SK (Tomlekova NB, Kozgar MI and Wani MR), , 2014. Induced mutagenesis: basic knowledge for technological success. Wageningen Acad Publ, Wageningen.

Datta SK and Chakrabarty D (Shu QY), 2009. Management of chimera and in vitro mutagenesis for development of new flower color/shape and chlorophyll variegated mutants in Chrysanthemum. Vienna, Austria, Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, International Atomic Energy Agency.

Devarumath RM, Nerkar GA, Farsangi FJ, Nikam AA and Harinath Babu K, 2015. Embracing biotechnology methods for crop improvement research in sugarcane. Current Status of Sugarcane Research in India. Nova Science Publishers, Inc. pp. 33–53.

Diao X and Jia G (Doust A and Diao X), 2017. Foxtail millet breeding in China. Switzerland, Springer International Publishing AG, Cham.

EFSA GMO Panel (EFSA Panel on Genetically Modified Organisms), Naegeli H, Bresson J-L, Dalmay T, Dewhurst IC, Epstein MM, Firbank LG, Guerche P, Hejatko J, Moreno FJ, Mullins E, Nogué F, Sánchez Serrano JJ, Savoini G, Veromann E, Veronesi F, Casacuberta J, Gennaro A, Paraskevopoulos K, Raffaello T and Rostoks N, 2020. Applicability of the EFSA Opinion on site-directed nucleases type 3 for the safety assessment of plants developed using site-directed nucleases type 1 and 2 and oligonucleotide-directed mutagenesis. EFSA Journal 2020;18(11):6299, 14 pp. https://doi.org/10.2903/j.efsa.2020.6299

EFSA (European Food Safety Authority), Martino L, Aiassa E, Halldórsson TI, Koutsoumanis PK; Naegeli H, Baert K, Baldinelli F, Devos Y, Lodi F, Lostia A, Manini P, Merten C, Messens W, Rizzi V, Tarazona J, Titz A and Vos S, 2020. Draft framework for protocol development for EFSA's scientific assessments. EFSA supporting publication 2020;EN-1843, 46 pp. https://doi.org/10.2903/sp.efsa.2020.EN-1843

Ehrenberg L, 1960. Induced mutation in plants: mechanisms and principles. Genetica Agraria, 12, 364–389.

FAO/IAEA, 2020. FAO/IAEA mutant variety database of the joint FAO/IAEA division of nuclear techniques in food and agriculture.

Favret EA, 1960. Induced mutations for resistance to diseases. Genetica Agraria, 13, 1–26.

Gustafsson A, 1960. Chemical mutagenesis in higher plants. Chemical mutagenesis. Lectures in memory of Erwin Baur I, 1959 organized by the Institute for Research on Cultivated Plants, Gatersleben, of the German Academy of Sciences at Berlin 26-28 July 195:14–29.

Hernández-Muñoz S, Pedraza-Santos ME, López PA, Gómez-Sanabria JM and Morales-García JL, 2019. Mutagenesis in the improvement of ornamental plants. Revista Chapingo, Serie Horticultura, 25, 151–167. https://doi.org/10.5154/r.rchsh.2018.12.022

Ibrahim R, Ahmad Z, Salleh S, Abu Hassan A and Ariffin S (VanHuylenbroeck J), 2018. Mutation breeding in ornamentals. Springer International Publishing Ag, Cham.

Jankowicz-Cieslak J and Till BJ, 2016a. Chemical mutagenesis and chimera dissolution in vegetatively propagated banana. Protocols, Springer International Publishing, Biotechnologies for Plant Mutation Breeding. pp. 39–54.

Jankowicz-Cieslak J and Till BJ, 2016b. Forward and reverse genetics in crop breeding. Advances in Plant Breeding Strategies: Breeding, Biotechnology and Molecular Tools. Springer International Publishing. pp. 215–240.

Jankowicz-Cieslak J, Mba C and Till BJ, 2016a. Mutagenesis for crop breeding and functional genomics. Protocols, Springer International Publishing, Biotechnologies for Plant Mutation Breeding. pp. 3–18.

Jankowicz-Cieslak J, Tai TH, Kumlehn J and Till BJ, 2016b. Biotechnologies for plant mutation breeding: Protocols. Springer International Publishing.

Kharkwal MC (Shu QY, Forster BP and Nakagawa H), 2012. A brief history of plant mutagenesis. Wallingford, UK, Cabi.

Kiunga FK, Kinyua M and Kiplagat O, 2014. Mutation breeding in Irish potatoes. RUFORUM Fourth Biennial Conference, Maputo, Mozambique, 19–25 July 2014:495–496.

Kodym A, Afza R, Forster BP, Ukai Y, Nakagawa H and Mba C (Shu QY, Forster BP and Nakagawa H), 2012. Methodology for physical and chemical mutagenic treatments. Wallingford, UK, Cabi.

Konzak CF, 1957. Genetic effects of radiation on higher plants. Quarterly Review of Biology, 27–45. https://doi.org/10.1086/401671

Kumawat S, Rana N, Bansal R, Vishwakarma G, Mehetre ST, Das BK, Kumar M, Kumar Yadav S, Sonah H, Raj Sharma T and Deshmukh R, 2019. Expanding avenue of fast neutron mediated mutagenesis for crop improvement. Plants, 8. https://doi.org/10.3390/plants8060164

Larkin PJ and Scowcroft WR, 1981. Somaclonal variation - a novel source of variability from cell cultures for plant improvement. TAG. Theoretical and Applied Genetics., 60, 197–214. https://doi.org/10.1007/bf02342540

Latado RR, Tulmann Neto A and Figueira A, 2012. *In vivo* and *in vitro* mutation breeding of citrus. Bioremediation, Biodiversity and Bioavailability, 6, 40–45.

Lee LS, Till BJ, Hill H, Huynh OA and Jankowicz-Cieslak J, 2014. Mutation and mutation screening. In: Henry RJ and Furtado A (eds.). Cereal Genomics: Methods and Protocols. Humana Press Inc, Totowa. pp. 77–95.

Leitao JM (Shu QY, Forster BP and Nakagawa H), 2012. Chemical mutagenesis. Wallingford, UK, Cabi.

Lundqvist U (Shu QY), 2009. Eighty years of Scandinavian barley mutation genetics and breeding. Vienna, Austria, Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, International Atomic Energy Agency.

Lundqvist U, 2014. Scandinavian mutation research in barley - a historical review. Hereditas (Lund), 151, 123–131. https://doi.org/10.1111/hrd2.00077

Mac Key J, 1956. Mutation breeding in Europe.

Maghuly F, Bado S, Jankowicz-Cieslak J and Laimer M, 2016. Chemical and physical mutagenesis in Jatropha curcas. Protocols, Springer International Publishing, Biotechnologies for Plant Mutation Breeding. pp. 21–38.

Magori S, Tanaka A, Kawaguchi M (Meksem K and Kahl G), 2010. Physically induced mutation: ion beam mutagenesis. Wiley-Blackwell, Chichester, UK.

Maluszynski M, Szarejko I, Bhatia CR, Nichterlein K, Lagoda PJL (Ceccarelli S, Guimaraes EP and Weltzien E), 2009. Methodologies for generating variability. Part 4: Mutation techniques. Food and Agriculture Organization of the United Nations (FAO), Rome, Italy.

Maluszynski M, Szarejko I, Maluszynska J and Szurman-Zubrzycka M, 2016. Mutation Techniques. Elsevier Inc, Encyclopedia of Applied Plant Sciences. pp. 215–228.

Manova V and Gruszka D, 2015. DNA damage and repair in plants – from models to crops. Frontiers in Plant Science, 6. https://doi.org/10.3389/fpls.2015.00885

Matsuo T, 1962. Review of research on use of radiation-induced mutations in crop breeding with special reference to rice in Japan. International Journal of Applied Radiation and Isotopes, 13, 477–478.

Mba C, 2013. Induced mutations unleash the potentials of plant genetic resources for food and agriculture. Agronomy-Basel, 3, 200–231. https://doi.org/10.3390/agronomy3010200

Mba C, Afza R, Bado S, Jain SM (Davey MR and Anthony P), 2010. Induced mutagenesis in plants using physical and chemical agents. Wiley-Blackwell, Chichester, UK.

Mou B, 2011. Mutations in lettuce improvement. International Journal of Plant Genomics, 2011. https://doi.org/10.1155/2011/723518

Muller HJ, 1927. Artificial transmutation of the gene. Science, 66, 84. https://doi.org/10.1126/science.66.1699.84

Nakagawa H (Shu QY), 2009. Induced mutations in plant breeding and biological researches in Japan. Vienna, Austria, Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, International Atomic Energy Agency.

Navjot K, Satinder K, Kuljit K and Vijay S, 2018. A review on mutation breeding for the improvement of crop plants: present and future. Agriways, 6, 50–58.

Nybom N, 1961. The use of induced mutations for the improvement of vegetatively propagated plants. pp. 252–294.

Pathirana R, 2011. Plant mutation breeding in agriculture. CAB Reviews: Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources, 6, 1–20. https://doi.org/10.1079/pavsnnr20116032

Phang I, Taylor HH and Leung DWM, 2012. Chemical mutagenesis for improving potential of plants to remediate environments with heavy metal contaminants. Research Trends, Trivandrum, India.

Rafiq Wani M, Imran Kozgar M, Tomlekova N, Khan S, Gul Kazi A, Ahmad Sheikh S and Ahmad P, 2014. Mutation breeding: A novel technique for genetic improvement of pulse crops particularly chickpea (Cicer arietinum L.). Springer, New York. pp. 217–248.

Riaz A and Gul A, 2015. Plant mutagenesis and crop improvement. Springer International Publishing, Crop Production and Global Environmental Issues. pp. 181–209.

Ryu JS, Choi JM, Kang YH and Kim SY (Khush GS, Brar DS and Hardy B), 2003. Developing blast-resistant lines in rice through tissue culture methods. Singapore, Singapore, World Scientific Publishing Co. Pte. Ltd.

Sakamoto AN, 2019. Translesion synthesis in plants: Ultraviolet resistance and beyond. Front Plant Sci, 10, 1208. https://doi.org/10.3389/fpls.2019.01208

Sen NK, 1951. Mutagenic chemicals. Science and Culture, 16, 480–481.

Sheela VL, Sheena A (Tomlekova NB, Kozgar MI and Wani MR), 2014. Novel trends and achievements in breeding of tropical ornamental crops especially orchids and anthuriums: the mutation breeding approach. Wageningen Acad Publ, Wageningen.

Shen Y, Pan G and Lübberstedt T, 2015. Haploid strategies for functional validation of plant genes. Trends in Biotechnology, 33, 611–620. https://doi.org/10.1016/j.tibtech.2015.07.005

Shu QY, Forster BP and Nakagawa H, 2012. Plant mutation breeding and biotechnology. CABI Publishing.

Singer SD, Laurie JD, Bilichak A, Kumar S and Singh J, 2021. Genetic variation and unintended risk in the context of old and new breeding techniques. Critical Reviews in Plant Sciences, 40, 68–108. https://doi.org/10.1080/07352689.2021.1883826

Spencer-Lopes MM, Forster BP, Jankuloski L (Spencer-Lopes MM, Forster BP and Jankuloski L), 2018. Manual on mutation breeding. Italy, Food and Agriculture Organization of the United Nations (FAO), Rome.

Stadler LJ, 1928. Mutations in barley induced by X-rays and radium. Science, 68, 186. https://doi.org/10.1126/science.68.1756.186

Suprasanna P, Patade VY, Desai NS, Devarumath RM, Kawar PG, Pagariya MC, Ganapathi A, Manickavasagam M and Babu KH, 2011. Biotechnological developments in sugarcane improvement: an overview. Sugar Tech, 13, 322–335. https://doi.org/10.1007/s12355-011-0103-3

Suprasanna P, Vitthal SB and Yadav PV, 2012. *In vitro* mutagenesis and selection in plant tissue cultures and their prospects for crop improvement. Bioremediation, Biodiversity and Bioavailability, 6, 6–14.

Suprasanna P, Mirajkar SJ, Patade VY, Jain SM (Tomlekova NB, Kozgar MI and Wani MR), 2014. Induced mutagenesis for improving plant abiotic stress tolerance. Wageningen Acad Publ, Wageningen.

Suprasanna P, Mirajkar SJ and Bhagwat SG, 2015. Induced mutations and crop improvement. Plant Diversity, Organization, Function and Improvement, Springer India, Plant Biology and Biotechnology. pp. 593–617.

Tadele Z, Mba C, Till BJ (Jain SM and Brar DS), 2010. TILLING for mutations in model plants and crops. Springer, Dordrecht.

Tanaka A and Hase Y, 2010. Applications to biotechnology: Ion-beam breeding of plants. Recent Advances, Applications, and Interfaces, CRC Press, Charged Particle and Photon Interactions with Matter. pp. 943–957.

Till BJ, Zerr T, Comai L, Henikoff S (Shu QY, Forster BP and Nakagawa H), 2012. A protocol for TILLING and eco-TILLING. Cabi, Wallingford, UK.

Till BJ, Datta S and Jankowicz-Cieslak J, 2018. TILLING: The next generation. Advances in Biochemical Engineering/Biotechnology. Place Springer Science and Business Media Deutschland GmbH, Springer Science and Business Media Deutschland GmbH. pp. 139–160.
Tomlekova N, Todorova V, Petkova V, Yancheva S, Nikolova V, Panchev I and Penchev E (Shu QY), 2009. Creation and evaluation of induced mutants and valuable tools for pepper breeding programmes. Vienna, Austria, Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, International Atomic Energy Agency.
Tuteja N, Ahmad P, Panda BB and Tuteja R, 2009. Genotoxic stress in plants: shedding light on DNA damage, repair and DNA repair helicases. Mutation Research - Reviews in Mutation Research, 681, 134–149. https://doi.org/10.1016/j.mrrev.2008.06.004
Viana VE, Pegoraro C, Busanello C and Costa de Oliveira A, 2019. Mutagenesis in rice: the basis for breeding a new super plant. Frontiers in Plant Science, 10. https://doi.org/10.3389/fpls.2019.01326
Wani MR, Kozgar MI, Tomlekova NB and Khan S (Tomlekova NB, Kozgar MI and Wani MR), 2014. Selection for polygenic variability in early mutant generations of mungbean (*Vigna radiata* (L.) Wilczek). Wageningen Acad Publ, Wageningen.

Abbreviations

BER	base excision repair
CPD	cyclobutane pyrimidine dimers
DEB	diepoxybutane
DES	diethyl sulfate
DH	double haploids
DMS	dimethyl sulfate
DNA	deoxyribonucleic acid
DSB	double strand break
EI	ethyleneimine
EMS	ethyl methanesulfonate
ENNG	1-ethyl-2-nitro-1-nitrosoguanidine
ENU (ENH)	*N*-ethyl-*N*-nitrosourea
FAO	Food and Agriculture Organization of the United Nations
FNB	fast neutron bombardment
GMO	genetically modified organism
GR_{30-60}	growth reduction between 30 and 60% of the treated plant material in generation M1
Gray (Gy)	the current SI unit of absorbed dose equivalent to 1 Joule per kg ($1Gy = 1Jkg^{-1}$)
HR	homologous recombination
IAEA	International Atomic Energy Agency
IBR	ion beam radiation
LD_{50}	lethal dose which kills 50% of the treated plant material.
LTE	linear energy transfer
MC WG	molecular characterisation working group
MMR	mismatch repair
MMS	methyl methanesulfonate
MNNG	*N*-methyl-*N1*-nitro-*N*-nitrosoguanidine
MNU (MNH)	*N*-methyl-*N*-nitrosourea
NDEA	*N*,*N*-diethylnitrous amide
NDMA	*N*,*N*-dimethylnitrous amide
NER	nucleotide excision repair
NHEJ	non-homologous end joining
PCR	polymerase chain reaction
6-4 PP	pyrimidine (6–4) pyrimidone [(6–4) PPs]
RNA	ribonucleic acid
SA	sodium azide
SSB	single strand break
TILLING	targeting Induced Local Lesions in Genomes
UV	ultraviolet

Glossary

Adenine	one of the nitrogenous bases composing the structure of the nucleotides
Chemical mutagenesis	the use of chemical mutagens in random mutagenesis.
Chimerism	sectoral differences in a mutant where cells of different genotypes exist side-by-side in the tissues of the same individual plant.
Genome	a complete set of genetic information in an organism
Genotype	the complete genetic make up of an organism. The complete set of genes of an organism
Guanine	one of the nitrogenous bases composing the structure of the nucleotides
Heterozygosity	the condition of having two different alleles at a locus.
Homozygosity	the condition of having two identical alleles at a locus
In vitro	a process which is performed or taking place outside a living organism, for example in test tubes or Petri dish.
In vivo	a process which is performed or taking place in a living organism
Induced mutation	changes in the DNA caused by mutagenic agents brought by humans
Mutation breeding	the process of increasing the genetic variability of plant species of agronomic interest by inducing mutations at a higher frequency compared to spontaneous processes, in order to identify and select valuable agronomic traits.
Mutation	changes in the genetic material of an organism that may be passed to the offspring
Nucleotide	the basic building block of nucleic acids
Phenotype	the observable physical properties of an organism. The phenotype is determined by the genotype in a given environment.
Physical mutagenesis	the use of physical mutagens in random mutagenesis
Ploidy	the number of complete set of chromosomes in a cell or an individual
Purine	a heterocyclic aromatic organic compound forming the backbone of guanine and adenine bases
Pyrimidine	a heterocyclic aromatic organic compound forming the structure of cytosine and thymine.
Random mutagenesis technique	a technique that results in the introduction of mutations in the genome in a non-targeted way
Replication	a molecular mechanism by which a double-stranded DNA molecule is copied to produce two identical DNA molecules
Somaclonal variation	the genetic diversity observed in progeny of plants regenerated from tissue cultures
Spontaneous mutation	changes in the DNA that are not caused by human intervention
Transcription	the molecular mechanism by which the information in a DNA strand is copied into a new messenger RNA (mRNA) molecule
Transposon	mobile DNA sequences capable of changing their position within a genome

Appendix A – Literature searches. Search strategies

CAB Abstracts

Date of the search 22/12/2020
Limits: reviews or books and English language

Set	Query	Results	Comments
# 19	#18 OR #12 OR #9 Indexes=CAB Abstracts Timespan=All years	334	Search 1 OR Search 2 OR Search 3
# 18	#17 OR #16 *Indexes=CAB Abstracts Timespan=All years*	33	Search 3: Mutagenesis in plants specific AND Techniques AND (Reviews OR Books)
# 17	#14 AND #13 Refined by: DOCUMENT TYPES: (BOOK CHAPTER) *Indexes=CAB Abstracts Timespan=All years*	12	Mutagenesis in plants specific AND Techniques AND Books
# 16	#15 AND #6 *Indexes=CAB Abstracts Timespan=All years*	24	Mutagenesis in plants specific AND Techniques AND Reviews
# 15	#14 AND #13 *Indexes=CAB Abstracts Timespan=All years*	174	Mutagenesis in plants specific AND Techniques
# 14	((TS=(technique* OR method* OR "in vivo" OR "in vitro" OR tool OR tools))) *AND* LANGUAGE: (English OR Unspecified) *Indexes=CAB Abstracts Timespan=All years*	3,195,661	Techniques
# 13	((TI=(mutagenesis OR ((gene OR genes OR genetic) AND mutation*)) AND TI=(plant OR plants OR crop OR crops OR seed OR seeds OR fruit OR fruits OR tree OR trees))) *AND* LANGUAGE: (English OR Unspecified) *Indexes=CAB Abstracts Timespan=All years*	431	Mutagenesis in plants specific [only title]
# 12	#11 AND #6 *Indexes=CAB Abstracts Timespan=All years*	3	Search 2: Random mutagenesis specific AND plants AND Reviews
# 11	#10 AND #1 *Indexes=CAB Abstracts Timespan=All years*	63	Random mutagenesis specific AND plants
# 10	((TI=((random OR conventional) AND (mutagen* OR ((gene OR genes OR genetic) AND mutation*))))) *AND* LANGUAGE: (English OR Unspecified) *Indexes=CAB Abstracts Timespan=All years*	148	Random mutagenesis specific [only title]
# 9	#8 OR #7 *Indexes=CAB Abstracts Timespan=All years*	309	Search 1: Chemical or physical mutagenesis 1 OR 2 AND plants AND (Books OR Reviews)
# 8	#4 AND #1 Refined by: DOCUMENT TYPES: (BOOK CHAPTER OR BOOK) *Indexes=CAB Abstracts Timespan=All years*	118	Chemical or physical mutagenesis 1 OR 2 AND plants AND Books
# 7	#6 AND #5 *Indexes=CAB Abstracts Timespan=All years*	215	Chemical or physical mutagenesis 1 OR 2 AND plants AND Reviews
# 6	(TS=(review* OR overview OR overviews OR "meta analys*" OR metanalys*)) *AND* LANGUAGE: (English OR Unspecified) *Indexes=CAB Abstracts Timespan=All years*	656,890	Reviews
# 5	#4 AND #1 *Indexes=CAB Abstracts Timespan=All years*	2,682	Chemical or physical mutagenesis 1 OR 2 AND plants
# 4	#3 OR #2 *Indexes=CAB Abstracts Timespan=All years*	3,278	Chemical or physical mutagenesis 1 OR 2

Set	Query	Results	Comments
# 3	(TS=((Chemical* OR physical*) NEAR/3 (mutagen OR mutagens OR mutagenesis OR mutation OR mutations))) *AND* LANGUAGE: (English OR Unspecified) *Indexes=CAB Abstracts Timespan=All years*	2,871	Chemical or physical mutagenesis 2
# 2	(TS=(((chemical* OR physical*) NEAR/3 (agent OR agents OR treatment*)) AND (mutagen OR mutagens OR mutagenesis OR mutation OR mutations))) *AND* LANGUAGE: (English OR Unspecified) *Indexes=CAB Abstracts Timespan=All years*	751	Chemical or physical mutagenesis 1
# 1	(TS=(plant OR plants OR crop OR crops OR seed OR seeds OR fruit OR fruits OR tree OR trees)) *AND* LANGUAGE: (English OR Unspecified) *Indexes=CAB Abstracts Timespan=All years*	5,361,889	Plants

Scopus

Date of the search 22/12/2020
Limits: reviews or books and English language

Set	Search Terms	Results	Comments
15	((TITLE ((mutagenesis OR ((gene OR genes OR genetic) AND mutation*)) AND (plant OR plants OR crop OR crops OR seed OR seeds OR fruit OR fruits OR tree OR trees))) AND (TITLE-ABS-KEY (technique* OR method* OR "in vivo" OR "in vitro" OR tool OR tools))) OR ((TITLE ((random OR conventional) AND (mutagen OR mutagens OR mutagenesis OR ((gene OR genes OR genetic) AND mutation*)))) AND (TITLE-ABS-KEY (plant OR plants OR crop OR crops OR seed OR seeds OR fruit OR fruits OR tree OR trees))) OR ((TITLE-ABS-KEY (plant OR plants OR crop OR crops OR seed OR seeds OR fruit OR fruits OR tree OR trees)) AND ((TITLE-ABS-KEY ((chemical* OR physical*) W/3 (mutagen OR mutagens OR mutagenesis OR mutation OR mutations))) OR (TITLE-ABS-KEY (((chemical* OR physical*) W/3 (agent OR agents OR treatment*)) AND (mutagen OR mutagens OR mutagenesis OR mutation OR mutations))))) AND (LIMIT-TO (LANGUAGE, "English")) AND (LIMIT-TO (DOCTYPE, "re") OR LIMIT-TO (DOCTYPE, "ch") OR LIMIT-TO (DOCTYPE, "bk"))	215 document results	Search 1 OR Search 2 OR Search 3
14	(TITLE ((mutagenesis OR ((gene OR genes OR genetic) AND mutation*)) AND (plant OR plants OR crop OR crops OR seed OR seeds OR fruit OR fruits OR tree OR trees))) AND (TITLE-ABS-KEY (technique* OR method* OR "in vivo" OR "in vitro" OR tool OR tools)) AND (LIMIT-TO (DOCTYPE, "re") OR LIMIT-TO (DOCTYPE, "ch") OR LIMIT-TO (DOCTYPE, "bk")) AND (LIMIT-TO (LANGUAGE, "English"))	33 document results	Search 3: Mutagenesis in plants specific AND Techniques AND (Book OR Reviews) AND language limit
13	(TITLE ((mutagenesis OR ((gene OR genes OR genetic) AND mutation*)) AND (plant OR plants OR crop OR crops OR seed OR seeds OR fruit OR fruits OR tree OR trees))) AND (TITLE-ABS-KEY (technique* OR method* OR "in vivo" OR "in vitro" OR tool OR tools))	164 document results	Mutagenesis in plants specific AND Techniques
12	TITLE-ABS-KEY (technique* OR method* OR "in vivo" OR "in vitro" OR tool OR tools)	26,103,275 document results	Techniques
11	TITLE ((mutagenesis OR ((gene OR genes OR genetic) AND mutation*)) AND (plant OR plants OR crop OR crops OR seed OR seeds OR fruit OR fruits OR tree OR trees))	407 document results	Mutagenesis in plants specific [only title]

Set	Search Terms	Results	Comments
10	(TITLE ((random OR conventional) AND (mutagen OR mutagens OR mutagenesis OR ((gene OR genes OR genetic) AND mutation*)))) AND (TITLE-ABS-KEY (plant OR plants OR crop OR crops OR seed OR seeds OR fruit OR fruits OR tree OR trees)) AND (LIMIT-TO (DOCTYPE, "re") OR LIMIT-TO (DOCTYPE, "ch")) AND (LIMIT-TO (LANGUAGE, "English"))	3 document results	Search 2: Random mutagenesis specific AND plants AND (Book OR Reviews) AND language limit
9	(TITLE ((random OR conventional) AND (mutagen OR mutagens OR mutagenesis OR ((gene OR genes OR genetic) AND mutation*)))) AND (TITLE-ABS-KEY (plant OR plants OR crop OR crops OR seed OR seeds OR fruit OR fruits OR tree OR trees))	44 document results	Random mutagenesis specific AND plants
8	TITLE ((random OR conventional) AND (mutagen OR mutagens OR mutagenesis OR ((gene OR genes OR genetic) AND mutation*)))	721 document results	Random mutagenesis specific [only title]
7	(TITLE-ABS-KEY (plant OR plants OR crop OR crops OR seed OR seeds OR fruit OR fruits OR tree OR trees)) AND ((TITLE-ABS-KEY ((chemical* OR physical*) W/3 (mutagen OR mutagens OR mutagenesis OR mutation OR mutations))) OR (TITLE-ABS-KEY (((chemical* OR physical*) W/3 (agent OR agents OR treatment*)) AND (mutagen OR mutagens OR mutagenesis OR mutation OR mutations)))) AND (LIMIT-TO (DOCTYPE, "re") OR LIMIT-TO (DOCTYPE, "ch") OR LIMIT-TO (DOCTYPE, "bk")) AND (LIMIT-TO (LANGUAGE, "English"))	191 document results	Search 1: Chemical OR physical mutagens OR agent mutagens AND plants AND (Books OR Reviews) AND language limit
6	(TITLE-ABS-KEY (plant OR plants OR crop OR crops OR seed OR seeds OR fruit OR fruits OR tree OR trees)) AND ((TITLE-ABS-KEY ((chemical* OR physical*) W/3 (mutagen OR mutagens OR mutagenesis OR mutation OR mutations))) OR (TITLE-ABS-KEY (((chemical* OR physical*) W/3 (agent OR agents OR treatment*)) AND (mutagen OR mutagens OR mutagenesis OR mutation OR mutations)))) AND (LIMIT-TO (DOCTYPE, "re") OR LIMIT-TO (DOCTYPE, "ch") OR LIMIT-TO (DOCTYPE, "bk"))	206 document results	Chemical OR physical mutagens OR agent mutagens AND plants AND (Books OR Reviews)
5	(TITLE-ABS-KEY (plant OR plants OR crop OR crops OR seed OR seeds OR fruit OR fruits OR tree OR trees)) AND ((TITLE-ABS-KEY ((chemical* OR physical*) W/3 (mutagen OR mutagens OR mutagenesis OR mutation OR mutations))) OR (TITLE-ABS-KEY (((chemical* OR physical*) W/3 (agent OR agents OR treatment*)) AND (mutagen OR mutagens OR mutagenesis OR mutation OR mutations))))	1,679 document results	Chemical OR physical mutagens OR agent mutagens AND plants
4	(TITLE-ABS-KEY ((chemical* OR physical*) W/3 (mutagen OR mutagens OR mutagenesis OR mutation OR mutations))) OR (TITLE-ABS-KEY (((chemical* OR physical*) W/3 (agent OR agents OR treatment*)) AND (mutagen OR mutagens OR mutagenesis OR mutation OR mutations)))	13,529 document results	Chemical OR physical mutagens OR agent mutagens
3	TITLE-ABS-KEY ((chemical* OR physical*) W/3 (mutagen OR mutagens OR mutagenesis OR mutation OR mutations))	11,813 document results	Chemical OR physical mutagens
2	TITLE-ABS-KEY (((chemical* OR physical*) W/3 (agent OR agents OR treatment*)) AND (mutagen OR mutagens OR mutagenesis OR mutation OR mutations))	2,174 document results	Chemical OR physical agent mutagens
1	TITLE-ABS-KEY (plant OR plants OR crop OR crops OR seed OR seeds OR fruit OR fruits OR tree OR trees)	3,776,442 document results	Plants

Web of Science. Core Collection

Date of the search 22/12/2020
Limits: reviews or books and English language

Set	Query	Results	Comments
# 17	#16 OR #11 OR #7 Indexes=SCI-EXPANDED, CPCI-S, BKCI-S, ESCI, CCR-EXPANDED, IC Timespan=All years	202	Search 1 OR Search 2 OR Search 3
# 16	#13 AND #12 Refined by: DOCUMENT TYPES: (REVIEW OR BOOK CHAPTER) AND LANGUAGES: (ENGLISH) Indexes=SCI-EXPANDED, CPCI-S, BKCI-S, ESCI, CCR-EXPANDED, IC Timespan=All years	18	Search 3: Mutagenesis in plants specific AND Techniques AND (Book OR Reviews) AND language limit
# 15	#13 AND #12 Refined by: DOCUMENT TYPES: (REVIEW OR BOOK CHAPTER) Indexes=SCI-EXPANDED, CPCI-S, BKCI-S, ESCI, CCR-EXPANDED, IC Timespan=All years	18	Mutagenesis in plants specific AND Techniques AND (Book OR Reviews)
# 14	#13 AND #12 Indexes=SCI-EXPANDED, CPCI-S, BKCI-S, ESCI, CCR-EXPANDED, IC Timespan=All years	119	Mutagenesis in plants specific AND Techniques
# 13	TS=(technique* OR method* OR "in vivo" OR "in vitro" OR tool OR tools) Indexes=SCI-EXPANDED, CPCI-S, BKCI-S, ESCI, CCR-EXPANDED, IC Timespan=All years	16,095,539	Techniques
# 12	(TI=(mutagenesis OR ((gene OR genes OR genetic) AND mutation*)) AND TI=(plant OR plants OR crop OR crops OR seed OR seeds OR fruit OR fruits OR tree OR trees)) Indexes=SCI-EXPANDED, CPCI-S, BKCI-S, ESCI, CCR-EXPANDED, IC Timespan=All years	393	Mutagenesis in plants specific [only title]
# 11	#8 AND #1 Refined by: DOCUMENT TYPES: (BOOK CHAPTER OR REVIEW) AND LANGUAGES: (ENGLISH) Indexes=SCI-EXPANDED, CPCI-S, BKCI-S, ESCI, CCR-EXPANDED, IC Timespan=All years	4	Search 2: Random mutagenesis specific AND plants AND (Book OR Reviews) AND language limit
# 10	#8 AND #1 Refined by: DOCUMENT TYPES: (BOOK CHAPTER OR REVIEW) Indexes=SCI-EXPANDED, CPCI-S, BKCI-S, ESCI, CCR-EXPANDED, IC Timespan=All years	4	Random mutagenesis specific AND plants AND (Book OR Reviews)
# 9	#8 AND #1 Indexes=SCI-EXPANDED, CPCI-S, BKCI-S, ESCI, CCR-EXPANDED, IC Timespan=All years	40	Random mutagenesis specific AND plants
# 8	(TI=((random OR conventional) AND (mutagen OR mutagens OR mutagenesis OR ((gene OR genes OR genetic) AND mutation*)))) Indexes=SCI-EXPANDED, CPCI-S, BKCI-S, ESCI, CCR-EXPANDED, IC Timespan=All years	804	Random mutagenesis specific [only title]
# 7	#4 AND #1 Refined by: DOCUMENT TYPES: (BOOK CHAPTER OR REVIEW) AND LANGUAGES: (ENGLISH) Indexes=SCI-EXPANDED, CPCI-S, BKCI-S, ESCI, CCR-EXPANDED, IC Timespan=All years	111	Search 1: Chemical OR physical mutagens OR agent mutagens AND plants AND (Books OR Reviews) AND language limit
# 6	#4 AND #1 Refined by: DOCUMENT TYPES: (BOOK CHAPTER OR REVIEW) Indexes=SCI-EXPANDED, CPCI-S, BKCI-S, ESCI, CCR-EXPANDED, IC Timespan=All years	112	Chemical OR physical mutagens OR agent mutagens AND plants AND (Books OR Reviews)

Set	Query	Results	Comments
# 5	#4 AND #1 Indexes=SCI-EXPANDED, CPCI-S, BKCI-S, ESCI, CCR-EXPANDED, IC Timespan=All years	842	Chemical OR physical mutagens OR agent mutagens AND plants
# 4	#3 OR #2 Indexes=SCI-EXPANDED, CPCI-S, BKCI-S, ESCI, CCR-EXPANDED, IC Timespan=All years	5,214	Chemical OR physical mutagens OR agent mutagens
# 3	TS=((Chemical* OR physical*) NEAR/3 (mutagen OR mutagens OR mutagenesis OR mutation OR mutations)) Indexes=SCI-EXPANDED, CPCI-S, BKCI-S, ESCI, CCR-EXPANDED, IC Timespan=All years	4,432	Chemical OR physical mutagens
# 2	TS=(((chemical* OR physical*) NEAR/3 (agent OR agents OR treatment*)) AND (mutagen OR mutagens OR mutagenesis OR mutation OR mutations)) Indexes=SCI-EXPANDED, CPCI-S, BKCI-S, ESCI, CCR-EXPANDED, IC Timespan=All years	964	Chemical OR physical agent mutagens
# 1	TS=(plant OR plants OR crop OR crops OR seed OR seeds OR fruit OR fruits OR tree OR trees) Indexes=SCI-EXPANDED, CPCI-S, BKCI-S, ESCI, CCR-EXPANDED, IC Timespan=All years	2,558,688	Plants